Effects of combined exposure to anti-androgens on development and sexual dimorphic behaviour in rats

Christiansen, Sofie

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PhD Thesis by

Sofie Christiansen

Department of Toxicology and Risk Assessment
National Food Institute, Technical University of Denmark

&

Department of Science, Systems and Models,
Roskilde University

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DATA SHEET

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Author: Sofie Christiansen.
Publisher: Technical University of Denmark
Affiliation: National Food Institute, Department of Toxicology and Risk Assessment
Address: Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
Telephone: + 45 72347025
Mobile phone: + 45 61265677
Fax: + 45 72347025
E-mail: sochr@food.dtu.dk

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Front cover: '*A rat pup in my hand is worth two in the bush'*
(Photo by: Mikkel Adsbøl for DTU)

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Preface

The present PhD project was performed at Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark. The two supervisors were Ulla Hass, National Food Institute, Technical University of Denmark, and Ole Andersen, Department of Science, Systems and Models, Roskilde University.

The thesis is divided into two parts. Part one includes a general introduction to the topic and the theoretical background for the work, an overview of the main results, a general discussion and the conclusions. Part two, appendix 1, presents the behavioural results in details whereas the other results are presented in published or submitted papers, or a manuscript in preparation.

Acknowledgements

A large number of persons have provided priceless contribution to the preparation of this thesis. I would hereby like to express my thankfulness to them all.

First I would like to thank my principal supervisor Ulla Hass, Department of Toxicology and Risk Assessment, for her support during the course of my study. She has been and will continuously be my mentor in reproductive toxicology. Thank you Ulla for your endless encouragement and optimism and thank you for believing in me!

I would also like to express gratitude to all my colleagues at the Department of Toxicology and Risk Assessment, the co-authors, and my supervisor at Roskilde University Ole Andersen, for their personal involvement and good advices throughout my PhD. A special thanks to Dorte Hansen who personally measured all the AGDs and counted all the nipples in the EDEN study. Thank you, for help with performing the behavioural studies to: Dorte Hansen, Bo Herbst, Lillian Sztuk, and Kenneth Rene Worm and for the dosing of the EDEN animals to: Elise Navntoft and Eva Ferdinandsen. Especially thanks to Pernille Hansen for being a competent replacement during my maternity leave. Thanks also to Per Nedergaard, DHI-group for his assistance with setting up the mating behaviour test.

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I would also like to thank the members of the "repro-& hormone group" at the department for the broadly scientific and cosy atmosphere and for interesting journal club discussions. Additionally, my two proofreaders Kirsten Pilegaard and Elsa Nielsen are thanked for their quick response and their linguistic enthusiasm.

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And last but not least, I would like to thank my family, my husband Philippe and our lovely "offspring" Sebastian and Mathilde for their support, patience and love.

Abbreviations and acronyms

AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
BBP	Benzylbutyl phthalate
Bw	Body weight
CONTAMED	Contaminant mixtures and human reproductive health - novel strategies for health impact and risk assessment of endocrine disrupters
DA	Dose addition
DBP	Di-*n*-butyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DHT	Dihydrotestosterone
EC_{50}	The median effective concentration
ED_{50}	The median effective dose
EDC	Endocrine disrupting chemical
EDEN	Exploring Novel Endpoints, Exposure, Low-dose- and Mixture-Effects in Humans, Aquatic Wildlife and Laboratory Animals (EU project)
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
FIN	Finasteride
GD	Gestational day
IA	Independent action
IPRA	Integrated Probabilistic Risk Assessment
LABC	Levator ani/bulbocavernosus muscles
LH	Luteinizing hormone
LOAEL	Lowest observed adverse effect level
MEHP	Mono-(2-ethylhexyl) phthalate
MNOAEL	Mixture no observed adverse effect level
NOAEL	No observed adverse effect level
NOEC	No observed effect concentration
NOEL	No observed effect level

NR	Nipple retention
NR NOAEL	No observed adverse effect level based on nipple retention
ODC	Ornithine decarboxylase
OECD	Organisation for Economic Co-operation and Development
PBP C3	Prostate binding protein subunit C3
PND	Postnatal day
PRO	Procymidon
PZ	Prochloraz
REACH	Registration, Evaluation and Authorisation of Chemicals
RPF	Relative Potency Factor
SAFE FOODS	Promoting Food Safety through a New Integrated Risk Analysis Approach for Foods
SRY	Sex determining region on the Y chromosome
TDF	The testis determination factor
TDI	Tolerable daily intake
TG	Test guideline
US EPA	United States Environmental Protection Agency
VIN	Vinclozolin

Summary

Background: Androgens are key regulators of male sexual differentiation during the *in utero* and early postnatal development. Exposure to endocrine disrupting chemicals (EDCs) that counteract androgen action at some stage in these periods can permanently demasculinise male foetuses and lead to malformations of the reproductive tract. The incidence of hypospadias (where the opening of the urethra is on the underside of the penis) in young boys has increased over the last decades but it is still unclear whether human exposure to endocrine disrupting chemicals may be responsible for this increase.

It is well-known that humans are exposed to a mixture of endocrine disrupting chemicals but risk assessment is currently based on the no observed adverse effect levels (NOAELs) for effects of one chemical at a time. The NOAEL is the highest tested dose at which no statistically or biologically adverse effects can be identified and is used in regulatory toxicology as a point of departure for establishing "acceptable" exposures for humans.

Purpose and methods: The overall purpose of this thesis is to explore the need for improving the future risk assessments of mixtures of EDCs. This is done by examining the following questions:

- Are there combined effects at NOAEL levels for individual anti-androgens based on the effects on anogenital distance, nipple retention and external malformations in male rats?
- Can the combined effects be predicted based on the model approaches; dose addition or independent action?
- Is sexually dimorphic behaviour in rats affected at lower dose levels of anti-androgens and thereby a more sensitive endpoint than morphological effects on the male external reproductive organs?

The thesis is based on the results of *in vivo* studies where mated female Wistar rats were exposed to anti-androgens either alone or in mixtures during pregnancy and lactation. The endpoints examined for anti-androgenic effects in the offspring were: Anogenital distance (AGD), nipple retention (NR), and external (morphological) malformations in pups and sexually mature male rats. Furthermore, the effects of the anti-androgens were studied in the offspring at different age period using several behavioural tests. Additionally, the development and use of a new test for mating behaviour was a part of this project.

Results and discussion: Results from the single chemical dose response studies showed that the NOAEL values found were very close to the NOAELs used by various regulatory bodies. It was also clear that DEHP (di-(2-ethylhexyl) phthalate) at a relatively low dose of 10 mg/kg bw/day caused adverse anti-androgenic effects on male rat development. The drug, finasteride was by far the most potent chemical, and it exhibited dose-response relationships with very shallow gradients for all endpoints.

In the mixture study with 3 similarly acting anti-androgens (vinclozolin, flutamide and procymidone), a combination of doses of each chemical, which on their own did not change the AGD statistically significantly, induced clear combination effects. Furthermore, exposure to low doses of the individual chemicals showed only modest effects on NR, while the mixture induced NR in the males that clearly approached the female values. Severe malformations of external genitalia (hypospadias) were observed in male rats with the mixture while the individual compounds did not cause any such effects. Increased frequencies (56%) of external malformations (hypospadias) were observed after exposure to a mixture of the three chemicals compared to administration of the three chemicals alone (0%). AGD was a good early biomarker, as a 25% reduction in mean AGD measured on postnatal day 1 was likely to result in clear malformations in approximately 50% and marked malformations in approximately 25% of the adult male rats. These combined effects could be predicted fairly accurately on the basis of information about the potency of the individual chemical components by using the dose addition concept.

Behaviour was as sensitive an endpoint as the morphological parameters (AGD, NR and malformations) in the mixture study with 3 similarly acting anti-androgens (vinclozolin, flutamide and procymidone). Results showed that learning was impaired in mixture exposed males when tested in the Morris water maze.

In the mixture study with 4 dissimilarly acting anti-androgens (vinclozolin, finasteride, DEHP and prochloraz) AGD, NR, and reproductive organ weights at PND (postnatal day) 16 were clearly affected in the mixture groups at dose levels where the individual chemicals caused no or only minor effects. The combined effects were equally well predicted by dose addition or independent action. The parameter ‘retained nipples’ was the most sensitive endpoint, with effects becoming noticeable at the lowest doses. Changes in AGD were almost as sensitive an endpoint, followed by reductions in prostate and LABC (levator ani/bulbocavernosus muscles) weights, and genital malformations. In addition, the experimentally observed responses for external malformations

clearly exceeded the predictions, suggesting that the combined effect of DEHP, vinclozolin, prochloraz and finasteride is synergistic with respect to malformations of external sex organs. To clarify, this thesis refers to effects which exceed expectations as synergism and those which meet expectations as additivity.

Behaviour was a less sensitive endpoint than the morphological parameters (AGD, NR and malformations) in the mixture study with 4 dissimilarly acting anti-androgens (vinclozolin, finasteride, DEHP and prochloraz).

Conclusion and perspectives: The data from the extended developmental toxicity studies with rats presented in this thesis suggest that dose addition models can in most cases predict the combined effects of anti-androgens and demonstrate that marked effects can occur at mixture doses below the NOAELs for the single chemicals.

Since unhindered androgen action is essential for human male development in foetal life, these findings are highly relevant to human risk assessment. This must be emphasised because the results clearly indicate that risk assessment based on NOAELs for single anti-androgens may underestimate the risk for hypospadias and other disruptions of male sexual differentiation.

Generally, behaviour was not as sensitive an endpoint as the as the development of the reproductive system but male behaviour may be just as sensitive an endpoint after exposure to mixtures of 3 androgen receptor (AR) antagonists (vinclozolin, flutamide, and procymidone). The learning behaviour in Morris water maze was in this study impaired at the same dose levels at which effects on NR and weights of epididymides, ventral prostate and bulbouretral glands were observed. Behavioural testing could therefore provide useful complementary information and contribute to a broader picture of the toxicity of the EDCs alone or in mixtures than studies that only take the development of reproductive organs into account.

Resumé (summary in Danish)

Baggrund: Androgener er væsentlige styrende hormoner i den seksuelle differentiering, der finder sted i fostertilstanden og i den tidlige postnatale udvikling hos hanner. Udsættelse for hormonforstyrrende stoffer, der modvirker androgener på et eller andet tidspunkt i disse perioder, kan permanent demaskulinisere hanfostre og føre til misdannelser af reproduktionsorganerne. Forekomsten af hypospadi (urinrøret udmunder på undersiden af penis) hos unge drenge er steget i de seneste årtier, men det er stadig uklart, om den humane eksponering for hormonforstyrrende stoffer kan være årsag til denne stigning.

Det er velkendt, at mennesker er udsat for adskillige hormonforstyrrende stoffer, men ved risikovurderinger af stoffer sammenlignes menneskers eksponering for et stof ad gangen normalt med stoffets NOAEL-værdi (”No Observed Adverse Effect Level ”), dvs. den højeste dosis af stoffet, som i dyreforsøg ikke har givet nogen skadelige effekter.

Formål og metoder: Det overordnede formål med denne afhandling er at undersøge behovet for at forbedre fremtidige risikovurderinger af udsættelse for blandinger af hormonforstyrrende stoffer. Dette gøres ved at undersøge følgende spørgsmål:

• Vil samtidig eksponering for hormonforstyrrende stoffer medføre samspilseffekter ved de enkelte stoffers NOAEL-værdier baseret på vurdering af effekter på anogenital afstand, bibeholdte brystvorter og ydre misdannelser hos hanrotter?

• Kan samspilseffekterne forudsiges baseret på modellerne ’dosis addition’ eller ’independent action’?

• Er seksuelt dimorf adfærd hos rotter påvirket ved lavere dosisniveauer af anti-androgener, og dermed en mere følsom parameter end morfologiske effekter på de ydre hanlige reproduktionsorganer?

Afhandlingen er baseret på resultaterne af *in vivo* undersøgelser, hvor parrede Wistar hunrotter blev doseret med anti-androgener enten som enkeltstoffer eller i blandinger i drægtigheds- og laktationsperioden. De effektmål, der generelt blev undersøgt for anti-androgene virkninger i afkommet var: Anogenital afstand (AGD), bibeholdelse af brystvorter (NR), og misdannelser af de ydre kønsorganer i unger og kønsmodne hanrotter. Derudover blev der undersøgt effekter på afkommet ved forskellige aldre i flere adfærdstests. I dette projekt indgik også udviklingen og brugen af en ny test for parringsadfærd.

Resultater og diskussion: Resultater fra dosis-respons forsøg med enkeltstoffer viste, at de NOAEL-værdier, der blev fundet, lå meget tæt på dem, der anvendes til regulatoriske formål. Det

blev også vist, at eksponering for DEHP (di-(2-ethylhexyl) phthalat) ved en relativt lav dosis på 10 mg/kg bw/dag førte til anti-androgene effekter hos hanrotter. Lægemidlet finasterid var væsentligt mere potent end de andre stoffer, og stoffet udviste dosis-respons-kurver med meget flad hældning for alle effektmål.

I det første kombinationsforsøg undersøgtes effekten af 3 anti-androgener, der alle blokerer androgenreceptoren (vinclozolin, flutamid og procymidon). Med hensyn til AGD medførte den samtidige eksponering for de 3 anti-androgener en tydelig samspilseffekt, der var mere markant end effekterne af de enkelte stoffer, der ikke førte til signifikante effekter på AGD. Lave doser af de enkelte kemikalier viste kun små effekter på NR, mens en kombination inducerede NR i hanrotter, der nærmede sig hunlige værdier.

Øget forekomst af misdannelser (hypospadi) (56%) blev observeret efter samtidig eksponering for de tre kemikalier i forhold til eksponering for de tre kemikalier alene (0%). AGD, målt PND 1, var en god tidlig biomarkør, idet en reduktion på 25% i AGD sandsynligvis vil resultere i alvorlige misdannelser af kønsorganerne hos ca. 50% og meget alvorlige misdannelser hos ca. 25% af de voksne hanrotter. Disse samspilseffekter kan forudsiges temmelig præcist på baggrund af viden om stoffernes potens og ved hjælp af konceptet om 'dosis addition'.

Adfærd var en lige så følsom parameter, som de morfologiske parametre (AGD, NR, misdannelser) i det første kombinationsforsøg. Her viste resultaterne, at indlæringen blev nedsat hos to grupper af kombinationseksponerede hanner, når de blev testet i en Morris water maze.

I det andet kombinationsforsøg undersøgtes effekten af 4 anti-androgener (vinclozolin, finasterid, DEHP og prochloraz) med forskellig virkningsmekanisme. AGD, bibeholdelse af brystvorter (NR), og vægt af reproduktionsorganer på PND (postnatal dag) 16 blev tydeligt påvirket af blandingen ved en dosis, hvor de enkelte kemikalier udviste ingen eller kun få effekter. De observerede effekter kunne forudsiges lige godt af 'dosis addition' såvel som af 'independent action'. Parameteren 'bibeholdelse af brystvorter' var det mest følsomme effektmål, med effekter ved de laveste doser. Ændringer i AGD var næsten lige så følsom, efterfulgt af vægtreduktioner i prostata og LABC (levator ani/bulbocavernosus muskler) og misdannelser.

Derudover oversteg de observerede resultater for misdannelser klart de forudsagte. Dette tyder på, at den samtidige eksponering af DEHP, vinclozolin, prochloraz og finasteride medførte synergistiske effekter med hensyn til misdannelser af ydre kønsorganer (hypospadi).

I denne afhandling anvendes betegnelsen 'synergi' når effekterne overstiger forventningerne, mens 'additivitet' anvendes når effekterne er lig de forventede.

Adfærd var i dette kombinationsforsøg (vinclozolin, finasterid, DEHP og prochloraz) ikke nær så følsom en parameter, som de morfologiske parametre (AGD, NR, misdannelser).

Konklusion og perspektiver: De resultater, der præsenteres i denne afhandling fra store studier med drægtige rotter og deres afkom, tyder på, at 'dosis additionsmodellen' i langt de fleste tilfælde kan forudsige samspilseffekten af anti-androgener, og viser at alvorlige effekter kan forekomme ved doser under NOAELs for enkeltstofferne.

Disse resultater er meget relevante for den humane risikovurdering, idet en uhindret androgen virkning er afgørende for den mandlige udvikling. Resultaterne viser klart, at risikovurdering baseret på NOAELs for de enkelte anti-androgener kan undervurdere risikoen for hypospadi og andre alvorlige forstyrrelser af den seksuelle differentiering.

Generelt var adfærden ikke nær så følsomt et parameter, som udviklingen af reproduktionssystemet (AGD, NR, misdannelser). Adfærd hos hanner efter eksponering for en kombination af 3 AR antagonister (vinclozolin, flutamid og procymidon) var dog ligeså følsomt idet indlæringsadfærd i Morris water maze i dette kombinationsforsøg var forringet ved samme dosisniveau, hvor bibeholdelse af brystvorter (NR), vægten af bitestikler, ventral prostata- og bulbouretral kirtler blev påvirket. Adfærdstestningen kan derfor give nyttige supplerende oplysninger og bidrage til et bredere billede af toksiciteten af anti-androgener alene eller i blandinger end undersøgelser, der kun tager udviklingen af reproduktionsorganer betragtning.

List of papers included

The present thesis is based upon the following 5 papers, referred to in the text by their roman numerals.

I **Christiansen S**, Boberg J, Axelstad M, Dalgaard M, Vinggaard AM, Metzdorff SB & Hass U. Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *In preparation.*

II Hass U, Scholze M, **Christiansen S**, Dalgaard M, Vinggaard AM, Axelstad M, Metzdorff SB & Kortenkamp A. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. Environmental Health Perspectives 15:122-128 (2007).

III Metzdorff SB, Dalgaard M, **Christiansen S**, Axelstad M, Hass U, Kiersgaard MK, Scholze M, Kortenkamp A & Vinggaard AM. Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after *in utero* exposure to antiandrogen mixtures. Toxicological Sciences 98:87-98 (2007).

IV **Christiansen S**, Scholze M, Axelstad M, Boberg J, Kortenkamp A & Hass U. Combined exposure to anti-androgens causes markedly increased frequencies of hypospadias in the rat. International Journal of Andrology 31:241-248 (2008).

V **Christiansen S**, Scholze M, Dalgaard M, Vinggaard AM, Axelstad M, Kortenkamp A & Hass U. Synergistic disruption of external male sex organ development by a mixture of four anti-androgens. *Submitted to Environmental Health Perspective.*

Scientific papers during my PhD period that provided important knowledge on effects of the single compounds include:

Vinggaard AM, **Christiansen S**, Laier P, Poulsen ME, Breinholt V, Jarfelt K, Jacobsen H, Dalgaard M, Nellemann C & Hass U. Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. Toxicological Sciences 85:886-897 (2005).

Laier P, Metzdorff SB, Borch J, Hagen ML, Hass U, **Christiansen S**, Axelstad M, Kledal T, Dalgaard M, McKinnell C, Brokken LJS & Vinggaard AM. Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. Toxicology and Applied Pharmacology 213:160-171 (2006).

Vinggaard AM, Hass U, Dalgaard M, Andersen HR, Jorgensen EB, **Christiansen S**, Laier P & Poulsen ME. Prochloraz: an imidazole fungicide with multiple mechanisms of action. International Journal of Andrology 29:186-192 (2006).

1. Introduction

In the last 10-15 years, increasing incidences of reproductive and developmental anomalies have been reported among wildlife and humans. The reproductive changes are seen in wildlife for example in alligators (Guillette, Jr. et al. 1994), female polar bears (Wiig et al. 1998) and male gulls (Fry 1995).

In humans it is well documented that the incidences of cryptorchidism and testicular cancer have increased over the last decades (Giwercman et al. 1993; Skakkebaek et al. 2001). The incidence of hypospadias is also rising, at least in Denmark, where studies have been performed (Boisen et al. 2005). Furthermore, since 1983 a decline in semen quality has been reported (Carlsen et al. 1992), and semen quality of young men in Northern Europe is generally quite poor (Jørgensen et al. 2006). However, this topic is controversial and other researchers question this decline (Fisch 2008). Anderson and colleagues conclude that based on these adverse trends in male reproductive health we may have reached a crucial "tipping point" towards male subfertility (Andersson et al. 2008).

The above-mentioned effects have been observed in several studies in experimental animal models showing reproductive malformations in male animals exposed to endocrine disrupters in the embryonic period (Gray et al. 1994; Gray et al. 1999b; Foster 2006; Welsh et al. 2008; Sharpe and Skakkebaek 2008). Studies have shown that the basic events of reproductive development are homologous in all mammalian species, and that rodent models have great utility for evaluating the potential of xenobiotics to alter human reproductive development (Gray 1992).

The aetiology of the above-mentioned reproduction related changes in humans are uncertain but man-made compounds detected in the environment are increasingly suspected to be involved (Gore 2007). The suspected compounds possess properties such as mimicking and/or antagonising (inhibiting) the action of normal hormones or affecting the synthesis or metabolism of endogenous hormones and their receptors (Sonnenschein and Soto 1998). They are often referred to as endocrine disrupting chemicals (EDCs).

The consequences of EDCs can be adverse and irreversible because of the crucial role that hormones play in controlling development (Gray and Kelce 1996). The intra-uterine development is a very delicate process as the reproductive and endocrine systems undergo complex changes in foetal life. Foetuses and newborns are therefore considered to be uniquely susceptible and vulnerable to influences of EDCs whereas similarly exposed young adults are only transiently affected (Gray 1992; O'Connor and Chapin 2003).

The following definitions of endocrine disrupters and potential endocrine disrupters have generally been accepted in the EU and are used throughout this thesis:
"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. A potential EDC is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism." (European Commission 1996)

EDEN was a large EU-project and is an acronym for "Exploring Novel Endpoints, Exposure, Low-dose- and Mixture Effects in Humans, Aquatic Wildlife and Laboratory Animals". The rationale for the EDEN project was to address key issues in the area of endocrine disruption. The National Food Institute took part in one of the themes, which focused on low-dose and combined effects of anti-androgens in rats.
Observations of low-dose effects of single endocrine disrupting chemicals have been debated (in the field of endocrine disruptors). Some have found effects at dose levels that are lower than normally tested in toxicology (vom Saal and Hughes 2005), while others have not been able to reproduce these low-dose effects (Ashby et al. 2004). In the EDEN dose response studies, low doses were included to find the NOAEL (No Observed Adverse Effect Level) for sensitive endpoints and to establish the shapes of the dose-response curves. Moreover, the established NOAEL doses for endocrine disrupting effects were used in the later mixture studies.
Humans are daily exposed to a range of multicomponent mixtures of toxicants, of which we do not know the effect (Backhaus et al. 2004) as toxicological research normally only examines effects of single compounds (Yang 1994). Generally, risk assessments and regulations in EU as well as other parts of the world are based on NOAELs for single chemicals, i.e., risk assessments are performed by a chemical-by-chemical approach and, do not take the cocktail of chemicals into account (Kortenkamp 2007).

2. Background

Chemicals are generally assessed for their risk for human health based on data from epidemiological studies, studies in laboratory animals and *in vitro* tests. Endocrine disrupting chemicals can show effects on the reproductive system and therefore reproductive toxicity studies are very relevant when it comes to these chemicals. There are several test guidelines (TG) for reproductive toxicity testing in the OECD program (OECD 2009) (Organisation for Economic Co-operation and Development). The OECD TG 440, "Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties" specifically screens for potential endocrine disruption, and the test guidelines for reproductive toxicity include some endocrine disrupting sensitive endpoints. The Hershberger assay and the Uterotrophic Bioassay are both new test guidelines and they have so far not become part of a testing strategy. Moreover, the development and validation of several *in vitro* test guidelines with endocrine disrupting endpoints is ongoing in OECD. However, endocrine disruption is a fairly new concept in risk assessment, and there are at present no test methods available, which specifically detect all effects that have been linked to the endocrine disruption mechanism(s) and mode(s) of action. The two-generation study (OECD TG 416) is currently the most complete study available. Both in this study and in the developmental toxicity study, OECD TG 414 (OECD TG 414), additional endocrine sensitive endpoints may be examined on a case-by-case basis when endocrine disruption is an issue of concern (Nielsen et al. 2008).

Assessment of anogenital distance (AGD), which is a sensitive endpoint for anti-androgenic effects in male offspring, is a parameter in the Two-Generation Reproduction Toxicity Study (OECD 2001), but is only measured in the second generation when triggered. Nipple retention in male offspring, which is another very sensitive endpoint for anti-androgenic effects in male offspring, is not included in any existing OECD guidelines.

Recently, an OECD expert group has started development of an OECD TG for an extended one-generation study based on the study design described in (Cooper et al. 2006). In the draft TG for the extended one-generation study, it is proposed to include assessment of AGD for each pup at PND 0, and retention of nipples in male pups at PND 12 or 13.

In the OECD TG 416, only one adult male is examined for malformations in the external sex organs (hypospadias). This endpoint is optimally observed in sexually mature male rats. As data have shown that it could be an advantage to increase the sensitivity of this endpoint it is discussed whether 2-3 adult male rats pr. litter should be included in future OECD test guidelines.

In the two-generation study, some effects on mating behaviour in the offspring may be observed, when the animals are mated to produce the second generation. However, in the extended one-generation, where the second generation only will be produced when triggered of findings in the first generation, the offspring is normally not mated, and effects on mating behaviour will therefore not be examined in animals that have been exposed during development.

Chemicals risk assessment is generally carried out by dealing with only single chemical at a time. The process does not take account of the likelihood that combination effects might occur when humans or wildlife come into contact with several agents at the same time. Thus, chemical mixtures and mixtures of EDCs are not considered by default when making a risk assessment. Different parts of the regulatory system are presently aware of the mixture issue, and several initiatives are taken in e.g. EFSA (European Food Safety Authority) and US National Academy of Science (see section 2.4).

2.1 Sexual differentiation

The foetus and young infant appear to be susceptible to endocrine-disrupting effects of xenobiotics. Exposure during critical developmental phases such as *in utero* and in the early postnatal period may lead to adverse effects on both reproductive development and neurodevelopment. The fact that many of the basic mechanisms underlying this developmental process are similar in all mammals indicates that chemicals that have adverse effects on reproductive development in rodents should be considered as potential human reproductive toxicants as well (Gray 1992).

2.1.1 Sexual differentiation of the reproductive system

In mammals the default sex/gender is female, as only exposure to androgens or testosterone of testicular origin during development will result in a male. In this manner androgens play a determining role during the most important periods of foetal development (Sharpe and Skakkebaek 2008).

Several endocrine disrupting chemicals can disturb this sexual differentiation, and thereby the developmental process of becoming a normal male or female. The primary step in the process of mammalian sexual differentiation occurs immediately after fertilisation. A sperm bearing either an X or a Y chromosome can fertilise an ovum. Whether the individual will develop testes or ovary is determined by the cellular expression of the testis determination factor (TDF), a protein encoded by

a gene known as SRY (sex determining region on the Y chromosome) (Berta et al. 1990; Welsh et al. 2008). When the SRY gene is expressed in the undifferentiated gonads, the testis determination factor binds to a specific hormone response element in the promotor region of the other genes that it regulates (Harley and Goodfellow 1994). When the protein products of these genes are produced, the male phenotypic traits, such as the testes, are formed (Berta et al. 1990). In the absence of expression of the SRY gene, the embryo develops into a female and the ovary is formed. In this respect, the female phenotype is considered the "default" pathway for reproductive development in mammals (O'Connor and Chapin 2003).

2.1.2 Sexual differentiation of the brain

An important part of the sexual differentiation that takes place during foetal and neonatal development is the sexual differentiation of the brain. This process, like the differentiation of the reproductive tract, is to a large extent determined by the levels of circulating sex hormones in the blood. In males, the testosterone secreted from the Leydig cells of the testes is secreted into the blood and reaches the brain. Testosterone is converted to oestradiol by aromatase in the brain and thereby oestradiol masculinises the male brain. In females, α-fetoprotein binds to oestradiol and prevents consequently oestradiol from entering the brain. This mechanism protects female brains from being masculinised by oestradiol (Puts DA et al. 2006).

During the 'critical periods' of development, the central nervous system is very sensitive to altered hormone levels as well as chemical exposures leading to severe and permanent effects on development, changing the course of sexual differentiation. Behavioural development and sexual behaviour in adult mammals are both influenced by, respectively, organizational and activational effects of testosterone. This is described in Figure 1 where a neonatal administration of testosterone to the female rat induces a "defeminisation pattern" of sexual behaviour (loss of female lordosis reflex). This effect is mediated by the aromatisation of testosterone in the brain: both testosterone and oestrogen appear to have a role in development of masculine copulatory behaviour (Forest 1983).

Reproductive behaviour patterns are not the only forms of behaviour that are sexually dimorphic. Gender differences have been observed in play behaviour, sweet preference, activity levels and spatial learning (Valenstein et al. 1967; Beatty 1979; MacLusky and Naftolin 1981; Hotchkiss et al. 2002; Kaya et al. 2002; Casto et al. 2003). These behaviours are not directly related to reproduction, but are influenced by gonadal steroids and can therefore be affected by altered levels of sex

hormones during development (Valenstein et al. 1967; Beatty 1979; MacLusky and Naftolin 1981; Hotchkiss et al. 2002; Kaya et al. 2002; Casto et al. 2003).

Exposure to EDCs in the critical periods of development can alter the sexual differentiation of the brain by interfering with the normal action of gonadal hormones (Schantz and Widholm 2001; Weiss 2002). However, little research has been done on the developmental neurotoxicity of anti-androgenic EDCs. The effects could go in two possible directions as it is expected that anti-androgens that lower the testosterone levels and/or inhibits the aromatase activity will contribute to a decreased conversion of testosterone into oestradiol, and thereby lower the oestradiol levels needed for complete behavioural masculinisation of the male rat brain. The result of this would be a demasculinised male.

Another expectation could be that the receptor mediated anti-androgens may block the androgen receptors in the brain, and thereby reduce the negative feedback that the endogenous testosterone exerts on the hypothalamus and pituitary, which will result in increased LH (luteinizing hormone) and testosterone production.

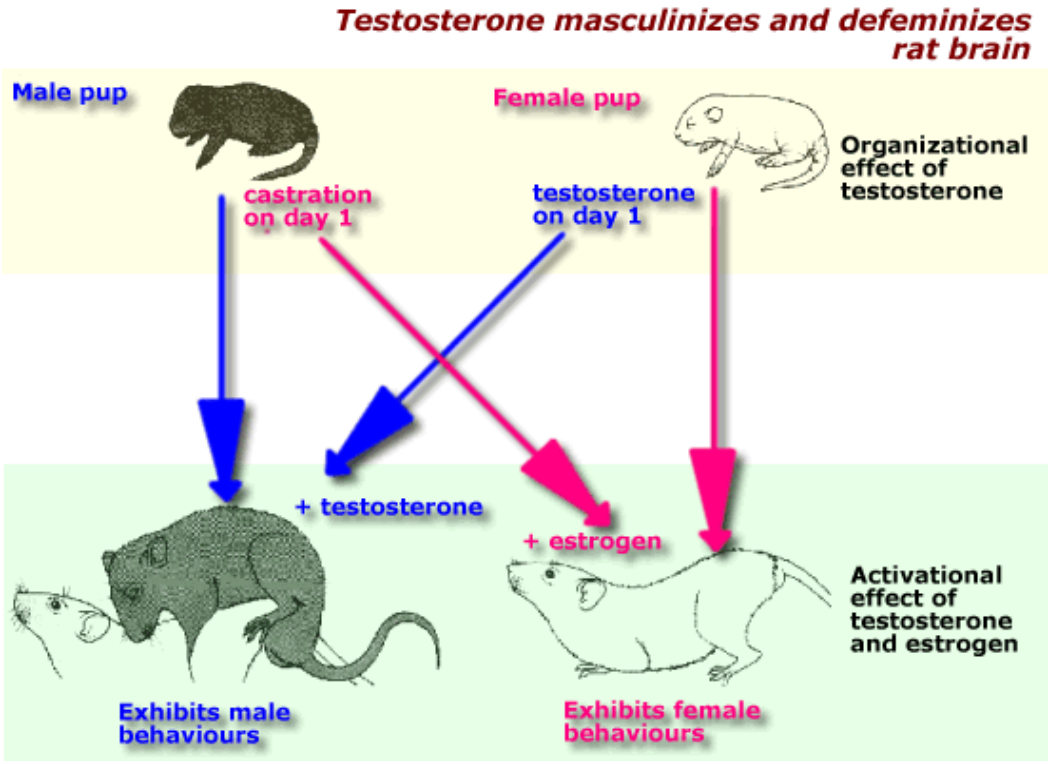

Figure 1. The masculinisation and feminisation of the rat brain. In the rat, it has been held that adult sexual behaviour depends solely on organizational effects. An intact male pup or a female pup treated with testosterone shortly after birth and given an injection of testosterone in adulthood will exhibit male behaviours (shown by blue arrows). An intact female pup or a neonatally castrated male pup given an injection of oestrogen in adulthood will exhibit female behaviours (shown by pink arrows). The Figure is used with permission from Dr. Paul Kenyon http://www.flyfishingdevon.co.uk/salmon.

2.2 DHT dependent tissue

Under the influence of the androgen dihydrotestosterone (DHT) the external genitalia develop in the male direction, while in the absence of androgens they develop in female direction (Imperato-McGinley et al. 1992). The specific development of the male external genitalia as well as the ventral prostate is DHT-dependent (Bowman et al. 2003). DHT is also responsible for regression of nipple anlagen in male rats, causing lack of nipple development (see 2.2.2). Therefore, male rats do not normally exhibit nipples or areolas as female offspring do. Furthermore, DHT is responsible for the development of external male genitalia and for growth of the perineum to produce a normal male AGD (Imperato-McGinley et al. 1985; Imperato-McGinley et al. 1986). The AGD is therefore a sensitive measure of prenatal anti-androgen exposure, and *in utero* exposure to anti-androgens can reduce the AGD and result in a diminished sexual dimorphism.

2.2.1 Anogenital distance (AGD)

Newborn male rats have no scrotum, and the external genitalia are undeveloped, and only a genital tubercle is apparent for both sexes. The AGD (Figure 2) is the distance from the anus to the insertion of this tubercle, the developing genital bud. The AGD is androgen dependent, and studies show that the AGD is normally about twice as long in male as in female rats. Similarly, in newborn humans the AGD measure was about two-fold greater in males than in females (Salazar-Martinez et al. 2004).

Significant correlation has been found between AGD and body weight in both rats ad humans. AGD has been reported alone and/or with the calculation of AGD/bw ratio, is therefore believed to be a more correct way to evaluate the results. It is also suggested that calculating the AGD/cube root of body weight is a better measurement since the body increases in 3 dimensions while AGD shows linear growth (Gallavan et al. 1999). Measuring the AGD in neonatal humans has been suggested as a non-invasive method to predict adult reproductive disorders (Welsh et al. 2008). Very recently, changes in AGD among male infants and suppressions of androgen synthesis have been associated with anti-androgen exposure in humans (Swan et al. 2005; Main et al. 2006).

Current regulatory guidelines for reproductive toxicity from US EPA (United States Environmental Protection Agency) and OECD (Organisation for Economic Co-operation and Development) explicitly define significant alterations of AGD as an adverse effect.

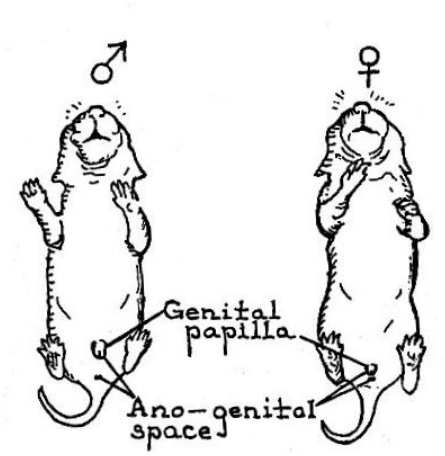

Fig.2 Anogenital distance (AGD) in rats (Farris 1949).

2.2.2 Nipple retention (NR)

Mammary gland development begins similarly in male and female rats; however, the further development of the nipple is sexually dimorphic (Kratochwil 1971). Female rats have nipples, whereas male rats possess only rudimentary mammary glands but no nipples. This is because locally produced DHT causes regression or apoptosis of the nipple anlagen in male rats (Imperato-McGinley et al. 1985; Imperato-McGinley et al. 1986). However, foetal exposure to anti-androgens can block this process, and the male offspring displays nipples similarly to their female littermates (Figure 3). Therefore, the retention of nipples in male rat pups is an indicator of impaired androgen action during the development.

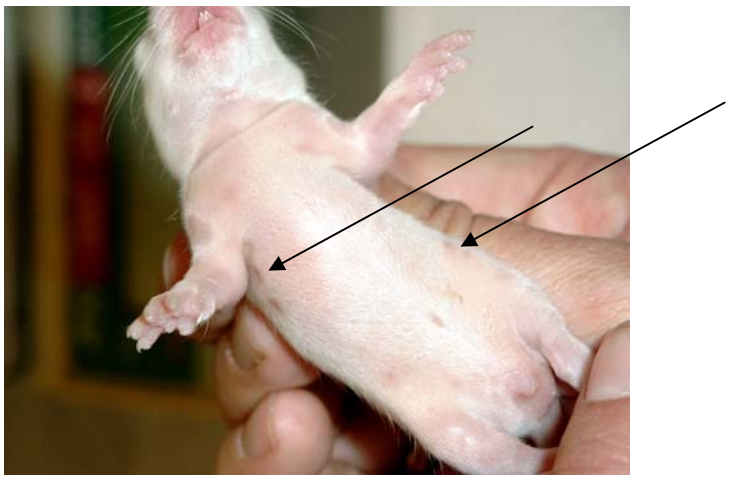

Figure 3. The arrows show the shadows from two retained nipples in an exposed male rat pup on PND 13 (Photo: Bo Herbst).

2.2.3 External malformations

Prenatal exposure to endocrine disrupting chemicals that interfere with the androgen signalling pathways during sexual differentiation can provoke a failure of urethral canalisation and fusion and results in hypospadias (Greek for "hole underneath").

Hypospadias in humans is one of the most common urogenital congenital anomalies affecting boys (Harris 1990). Prevalence estimates in Europe range from 4 to 24 per 10,000 births, depending on definition (Dolk et al. 2004) with higher rates of about 5% reported in a Danish study (Boisen et al. 2005). Little is known about the aetiology of hypospadias, but a role for EDCs have been proposed, and especially the anti-androgenic EDCs (Baskin et al. 2001).

The pattern of malformations in the male depends upon the specific mechanism of action of the toxicant, the dosage level administered and the timing of administration during pregnancy (Gray et al. 2004). Penile and scrotal fusion may be analogous to palatal shelf and neural tube fusion, in that the latter embryonic processes require embryonic cell movements and apposition of the two advancing tissues (Gupta and Goldman 1986).

Hypospadias in male rats (Figure 4) has been related to maternal exposure to the following substances:

1. Potent oestrogens or oestrogenic compounds (e.g. diethylstilbestrol DES)
2. Compounds that inhibit 5α-reductase (e.g. finasteride)
3. Drugs, herbicides, and dicarboximide and conazole fungicides that act as androgen receptor (AR) antagonists (e.g. flutamide, vinclozolin, procymidone, prochloraz)
4. Drugs, herbicides and conazole fungicides that inhibit cytochrome P450 enzymes involved in steroid hormone synthesis
5. Phthalate diesters inducing hypospadias by altering foetal testis Leydig cell differentiation which results in reduced steroid and peptide hormone production (e.g. di-*n*-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), and benzylbutyl phthalate (BBP).
6. Compounds that disrupt androgen action by inhibiting the conversion of progesterone to testosterone and thereby induce increased testicular progesterone concentrations in male rat foetuses (e.g. prochloraz) (Willingham et al. 2006; Agras et al. 2007).

The background incidences of malformations like hypospadias, spontaneous sex reversals, and / or agenesis of sex accessory and epididymal tissues are infinitesimally low in the male laboratory rat, if it occurs at all (Gray et al. 2004).

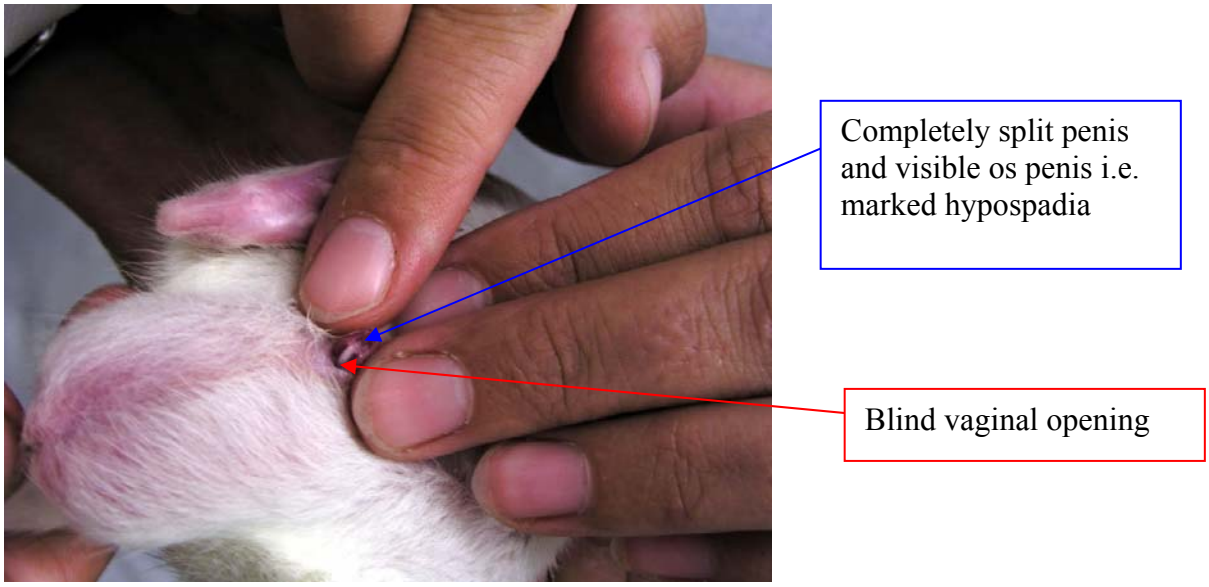

Figure 4. Malformations in the male rat (Photo: Bo Herbst)

2.3 The anti-androgens studied and their mechanisms of action

Androgen (from Greek *andros*, man and *gennaien*, to produce) is the name given to any substance, which induces the development of the male reproductive tract. Below is a presentation of the 6 anti-androgens studied in the EDEN project. The studies were model studies investigating a principle, and the anti-androgens were selected because they can disrupt male sexual differentiation in different ways, by a variety of mechanisms.

Vinclozolin is a common fungicide used in vineyards. It acts by inhibiting the spore germination in fungi. Vinclozolin metabolites, M1 and M2 bind to mammalian androgen receptors and act as AR antagonists, inhibiting androgen-dependent gene expression *in vivo* and *in vitro* by inhibiting AR-binding to DNA. Vinclozolin-treated male rat offspring display female-like AGD at birth, retained nipples, and cleft phallus with hypospadias, undescended testes (cryptorchidism), vaginal pouch, epididymal granulomas and small to absent sex accessory glands when exposed to higher doses (Gray et al. 1994; Kelce et al. 1994; Gray et al. 1999a; Hellwig et al. 2000). Some dose-response curves for vinclozolin exposed rats appear linear in the low dose range but fail to display an obvious threshold for AGD, NR and ventral prostate weight while induction of hypospadias and other malformations display apparent thresholds (Gray et al. 1997).

Procymidone is a dicarboximide fungicide, which appears to display a nearly identical toxicity profile as vinclozolin *in vitro* and *in vivo*, but is 2 fold less potent. It is also an AR antagonist, and a study in rats exposed perinatally to procymidone has shown decreased AGD, increased NR, higher incidence of hypospadias and cryptorchidism, and reduced prostate, testis and epididymal weights (Ostby et al. 1999).

Flutamide (4´-nitro-3'-trifluoromethyl-isobutyranilide) is a potent non-steroidal drug and AR antagonist primarily used to treat human prostate cancer. Studies in rats have demonstrated that pre- and postnatal flutamide exposure alter reproductive physiology by incomplete masculinisation of the exposed males (Imperato-McGinley et al. 1992). These effects are directly related to the anti-androgenic properties of flutamide - by competitively binding to the androgen receptors, and altering subsequent gene expression (Kelce et al. 1997). Flutamide inhibits testosterone from exerting its masculinising effects on sexual differentiation. Flutamide appears to be at least tenfold as potent as procymidone and vinclozolin (Gray et al. 2004). Studies with prenatal flutamide exposure have shown reduced AGD and increased NR, prostate agenesis and hypospadias in male rats (Hib and Ponzio 1995; McIntyre et al. 2001; Miyata et al. 2002).

DEHP is an abbreviation of di-(2-ethylhexyl) phthalate. DEHP is produced in a very high industrial volume and is used as a plasticizer in a wide range of consumer products, including vinyl floors, medical products and toys, making it one of the phthalates most abundantly found in the environment (Hellwig and Jäckh 1997; Koch et al. 2003; Müller et al. 2003). Several studies have shown that male rats exposed to DEHP during development have reduced AGD, retained nipples, reduced organ weights and cryptorchidism (Parks et al. 2000; Gray et al. 2000; Moore et al. 2001; Jarfelt et al. 2005). The most likely mechanism by which DEHP exerts its demasculinising effects seems to be reduction of testosterone production towards female levels in male rat foetuses during critical stages of sex differentiation (Parks et al. 2000; Borch et al. 2004; Wilson et al. 2004; Borch et al. 2006). Whether DEHP and the metabolite MEHP also act as anti-androgens by antagonising the AR receptor is still debated. Some *in vitro* studies have shown that neither DEHP nor MEHP are androgen receptor antagonists, as they do not compete with androgens for binding to the AR at concentrations up to 10 μM (Parks et al. 2000; Takeuchi et al. 2005) while one *in vitro* study has shown that metabolites of MEHP do act as AR antagonists (Stroheker et al. 2005).

Finasteride is a synthetic anti-androgen, used in humans as a treatment in benign prostatic hyperplasia in low doses and prostate cancer in higher doses. Finasteride acts by inhibiting 5α-reductase, and thereby blocking the conversion of testosterone to DHT. Studies in rats have shown reduced AGD, increased number of nipples and increased incidence of hypospadias in prenatally exposed male rats and feminisation of the external genitalia (Clark et al. 1990; Imperato-McGinley et al. 1992; Clark et al. 1993; Hib and Ponzio 1995).

Prochloraz is an imidazole fungicide, which has the potential to interfere with several mechanisms leading to effects on various organ systems (Vinggaard et al. 2005; Vinggaard et al. 2006). *In vitro*, prochloraz possesses anti-oestrogenic and anti-androgenic effects, and inhibits the activity of the oestrogen synthesising enzyme aromatase and activates the aryl hydrocarbon receptor (AhR) (Andersen et al. 2002; Vinggaard et al. 2002). The AhR (or dioxin receptor) is an intracellular receptor that mediates the toxic responses of dioxin and related chemicals. *In vivo*, prochloraz has caused an increased pregnancy length and induced incompletely masculinised male rat offspring after perinatal exposure (Vinggaard et al. 2005). Prochloraz is also able to induce increased testicular progesterone concentrations in male rat foetuses (Vinggaard et al. 2005; Laier et al. 2006; Blystone et al. 2007). Also a decreased level of testosterone in both testis and plasma was found in several studies as prochloraz inhibits the gonadal steroidogenesis (Laier et al. 2006; Blystone et al. 2007).
Furthermore, prochloraz decreased the concentration of thyroxin (T_4) and thyroid stimulating hormone (TSH) in serum of exposed rats (Vinggaard et al. 2005) indicating interference with the thyroid function. Studies have shown that male rats exposed to prochloraz during development show a reduced AGD, retained nipples, and mild dysgenesis of the male external genitalia. Moreover, an increased AGD indicating virilisation of the in female pups has been observed (Laier et al. 2006).

2.4 Mixture studies and prediction of combination effects

The prediction of combined effects of chemicals is complicated but some models have been developed for facilitating this. In the EDEN studies, we have developed an experimental setup that for most endpoints enables us to compare the observed mixture toxicity with the calculated effects using two models: dose addition (DA) and independent action (IA) (Kortenkamp and Altenburger 1998).

DA and IA are based on different assumptions and are developed separately. However, both models assume that the toxicants in the mixture do not influence the toxicity of the other toxicants or that there are no interactions in the mixture, and that the toxicants' modes of actions are known (Merino-García et al. 2003). Both concepts allow calculating expected mixture toxicity on the basis of known toxicities of the original mixture components (Backhaus et al. 2004).
Dose addition (DA) is often referred to as concentration addition, simple similar action or Loewe additivity (Loewe and Muischnek 1926). The concept has been introduced by Loewe and Muischnek in 1926, and the model assumes that all the chemicals in the mixture act on the same biological site (receptor or target organ), by the same mechanism of action, and that they differ only in their individual potency (Backhaus et al. 2004). The additive effects are described mathematically by summing up the doses of the individual chemicals in a mixture adjusted for their differences in potencies. The effect can be kept constant when one toxicant is replaced with an equal fraction of an equi-effective toxicant and in that way, the compounds in the mixture act as if they are dilutions of each other (Kortenkamp and Altenburger 1998). Combination effects based on dose addition should also result from toxicants at or below no observed effect levels (NOELs), provided sufficiently large numbers of components sum up to a suitably high total effect dose (Kortenkamp et al. 2007).

The equation for dose addition (4 components) is:

$$\mathrm{EDx}_{\mathrm{mixture}} = \left(\frac{p_1}{\mathrm{EDx}_1} + \frac{p_2}{\mathrm{EDx}_2} + \frac{p_3}{\mathrm{EDx}_3} + \frac{p_4}{\mathrm{EDx}_4} \right)^{-1}$$

Here, EDx_1, EDx_2, EDx_3 and EDx_4 are the effect doses of four chemicals that on their own produce the same quantitative effect x as the mixture, and p_1, p_2, p_3 and p_4 are the relative proportions of the corresponding individual doses present in the total mixture dose ("fraction in mixture").

The concept independent action (IA) is also known as simple dissimilar action, response addition, effect multiplication or Bliss independence, and was first applied by Bliss in 1939 (Bliss 1939). IA is based on the assumption that the toxic actions of each mixture compounds act on different sub-systems in organisms and do not interfere with each other but contribute to a common result (Reffstrup Klein 2002). IA can only describe the toxicity of mixtures that are composed of dissimilarly acting substances and IA is often considered to be the proper reference model when using dissimilar acting compounds in a mixture (Backhaus et al. 2004).

The equation for Independent action (4 components) is:

$$E(c_{mix}) = 1 - (1 - E(c_1)) \bullet (1 - E(c_2)) \bullet (1 - E(c_3)) \bullet (1 - E(c_4))$$

Here, $E(c_1)$, $E(c_2)$, $E(c_3)$, and $E(c_4)$ denote the fractional effects (x %) caused by the individual concentrations c_1, c_2, c_3, and c_4 of the four chemicals respectively, and $E(c_{mix})$ is the total effect of the mixture concentration c_{mix}. The individual effects of mixture compounds $E(c_i)$ are estimated from the concentration response functions determined for single substances.

This central principle of the concept of independent action is commonly taken to mean that exposed subjects are protected from mixture effects as long as the doses of all agents in the combination do not exceed their NOAELs. However, this only holds true if the NOAELs are zero effect levels but not if the there are small effects, even though statistically insignificant effects at the NOAELs (Kortenkamp et al. 2007). For example, in a case where 4 chemicals induce a statistically insignificant decrease of 4% of AGD the expected mixture effect is

$E(C_{mix}) = 1 - (1-0.04)^4 = 15\%$ (statistically significant)

In cases where the chemicals in the mixture each induce only small effects and there are a large number of chemicals in the mixture, the predictions based on dose addition and independent action will both predict a mixture effect. With a limited number of chemicals in the mixture and low doses of each chemical, the predictions based on dose addition and independent action may become quantitatively similar (Martin Scholze pers. com).
Several studies have examined the applicability of dose addition. Silva et al. examined multi-component mixtures of xenooestrogens in a yeast oestrogen-screen (E-screen) (Silva et al. 2002). The eight compounds were below their individual NOEC (no observed effect concentration) and they used reference models to predict their results. The results showed that DA was the most accurate method for prediction of the combination effects. Moreover they found that IA and effect summation led to a clear underestimation of the potential combination effects.
A similar pattern was found in a study where 16 similar and specific toxicants were mixed (Altenburger et al. 2000). The chemicals were phenol derivatives anticipated to have a common mode of action, and the results showed that the observed mixture toxicity was rather well predicted

by both DA and IA concepts. Dose addition showed an excellent predictive power while independent action, in contrast, underestimated the EC_{50}-values (the median effective concentration) of the mixture by a factor of a little more than three.

Faust and colleagues tested 16 dissimilarly acting chemicals in tests with freshwater algae. In that test system, the concept of dose addition tended to overestimate the combined effect of dissimilarly acting chemicals, while the concept of independent action provided a more accurate prediction (Faust et al. 2003). There exists some dispute in the understanding of "similar action", which makes it difficult to group the EDCs. In some cases, similar is regarded as an identical molecular mechanism of action at the same substructure of a specific receptor or a "same site of primary action" (Calamari and Vighi 1992). Others find it sufficient if similar action for mixture toxicities is seen as a similar mode of action (e.g. effects on growth or reproduction) (Hermens et al. 1984).

In the EDEN studies described in this thesis, the concept "similar action" is used literally. Our similarly acting chemicals were androgen receptor (AR) antagonists, while the dissimilarly acting chemicals have different mechanisms (AR antagonist, 5α-reductase inhibitor, suppress testosterone synthesis) and thus, all of the chemicals in the mixture studies acted as anti-androgens disrupting male sexual development.

Chemical mixtures and mixtures of EDCs are as earlier mentioned not considered by default when making a risk assessment. Several moves are underway to design approaches that take possible combination effects into consideration. A step in this direction has been taken in the EU research project SAFE FOODS (Promoting Food Safety through a New Integrated Risk Analysis Approach for Foods) in which an approach for a probabilistic cumulative risk assessment of anti-androgenic pesticides in foods has been presented ((Müller et al. 2009), submitted).

The approach is based on an Integrated Probabilistic Risk Assessment (IPRA) model for single substances (Van der Voet and Slob 2007) and extended to a cumulative risk assessment for 3 anti-androgenic pesticides (vinclozolin, procymidone and prochloraz) by using the Relative Potency Factor (RPF) approach (Larsen 2003). RPFs were estimated for several male reproductive endpoints using dose-response data from *in utero* exposure studies performed in our laboratory (Laier et al. 2006; Hass et al. 2007; Metzdorff et al. 2007).

In EFSA (European Food Safety Authority), one of the scientific panels is working on a cumulative risk assessment for a group of azolfungicides but the report is not yet published.

A very important recent example is the establishment of a US National Academy of Sciences committee on cumulative risk assessment for phthalates and related chemicals, at the request of the

US EPA. The report of this committee has appeared in December 2008 and in the summary of this report is stated: "*Thus, the evidence supports the use of dose-addition as an approximation in estimating cumulative risk posed by phthalates and other anti-androgens. The use of a dose-addition model is also supported by data that show cumulative effects at doses at which individual mixture components did not induce observable effects*" and moreover; "*Cumulative risk assessment based on common adverse outcomes is a feasible and physiologically relevant approach to the evaluation of the multiplicity of human exposures and directly reflects EPA's mission to protect human health*" (NRC 2008).

Wittassek and Angerer have made some metabolism studies in humans and conclude that the concept of a cumulative TDI (tolerable daily intake) value may be more appropriate for the consideration of the overall exposure and the potential human health risks resulting from everyday and simultaneous exposure to several phthalates (Wittassek and Angerer 2008).

In the new EU chemical legislation REACH (Registration, Evaluation and Authorisation of Chemicals), not much is written on combined exposure and mixtures. However it is stated about combined exposure: "*In special cases, where exposure occurs to a substance as well as to several very closely related and similar acting chemical substances (e.g. different salts of a metal or closely related derivatives of organic substances), the exposure evaluation and risk characterisation should reflect this aspect. If data are available, the exposure assessment should also include a scenario concerning this combined exposure. One way to conduct risk characterisation for combined exposure to closely related analogues could be to add exposures and to use a toxicological descriptor from a representative substance among the analogues. If data do not allow for a quantitative assessment, an attempt should be made to address the issue in a qualitative way*" (REACH part E) (ECHA 2008).

3. The overall purposes of this thesis

The overall purpose of this thesis is to explore the need for improving the future risk assessments of mixtures of EDCs. This is done by examining the following questions:

- Are there combined effects at NOAEL levels for individual anti-androgens based on the effects on anogenital distance, nipple retention and external malformations in male rats?
- Can the combined effects be predicted based on the model approaches; dose addition or independent action?
- Is sexually dimorphic behaviour in rats affected at lower dose levels of anti-androgens and thereby a more sensitive endpoint than morphological effects on the male external reproductive organs?

4. Experimental setup

An overview of the 5 *in vivo* studies included in this thesis is given in Table 1. In all of the studies (1 to 5), time-mated nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy. They were dosed by gavage from early gestation GD 7 until the day before expected birth, and then from PND 1 until PND 16 to cover the most sensitive periods of the development of the reproductive system. The studies were performed using 3-4 blocks (with one week in between), and all dose groups were equally represented in the blocks. Details about the laboratory animal housing and studies are reported in Papers I-V.

Study 1-3 was range finding and dose-response studies of the six chemicals (vinclozolin, flutamide, prochloraz, DEHP, procymidone, finasteride). Study 4 was a mixture study with exposures of 3 AR antagonists (vinclozolin, flutamide and procymidone) and study 5 was a mixture study with 4 dissimilarly acting anti-androgens (vinclozolin, finasteride, DEHP and prochloraz).

The endpoints AGD, NR, dysgenesis and malformations in the male rats were recorded by the same technician who was blinded with respect to exposure groups as mentioned in details in papers I-V. AGD and NR was recorded on all pups from all litters (i.e. around 4-5 males and 4-5 females pr. litter). The measurements on PND 16 were recorded in all male pups, which were not weaned for malformation endpoints and behavioural measurements. The malformations were recorded in 1 adult male per. litter in the following groups: Control, MIX 7.9, MIX 19.7, MIX 39.3, MIX 70.8, VIN 24.5 and VIN 95.9 mg/kg bw/day in the first study and all groups in the second mixture study. In both mixture studies the "fixed mixture ratio design" was used as the doses were combined at a fixed ratio based on the potencies of the individual chemicals. This approach was chosen in order to avoid that one single chemical contributed disproportionately to the overall mixture effect.

In the first mixture study the ratio of vinclozolin, flutamide, and procymidone was 31:1:18 and in the second mixture study the ratio of vinclozolin: finasteride: DEHP: prochloraz was 5:0.01:3:5. The mixture ration was based on ED50 for nipple retention in males. The choice of doses in the first mixture study was based on the concentration range described by the additivity prediction while the individual effect doses were derived from the dose response functions for the individual chemicals (paper II).

The choice of doses in the second mixture study was based on NOAELs as the lowest mixture (mix1) dose was equivalent to the sum of the chemicals' individual NOAELs for retained nipples

(NR NOAEL), the middle dose (mix2) was 5-fold higher, and the highest dose (mix3) 10-fold this values (paper V).

Study (No.) Paper	**Groups and doses All dams exposed from GD 7-PND 16**	**No. mated per group (pregnant*)**
(1) Vinclozolin and Flutamide, dose-response Prochloraz range finding **(Paper II, III, IV and V)**	Control: vehicle-dosed Vinclozolin: 5, 10, 20, 40, 80 or 160 mg/kg bw/day Flutamide: 0.5, 1, 2, 4, 8 or 16 mg/kg bw/day Prochloraz; 50 or 150 mg/kg bw/day	16 (13) 8 (6-8) 8 (6-8) 8 (6-8)
(2) Finasteride and DEHP, dose-response Procymidone range finding **(Paper I, V)**	**Control**: vehicle-dosed Finasteride: 0.001, 0.01, **0.1, 1, 10** or 100[a] mg/kg bw/day DEHP: 10, 30, 100, 300, 600 or 900 mg/kg bw/day Procymidone: 25 or 200 mg/kg bw/day	16 (15) 8/10 (7-9) 8(6-8) 4(2-3)
(3) Prochloraz, Procymidone and DEHP, dose-response **(Paper I, V)**	**Control**: vehicle-dosed Prochloraz: 5, **10, 25, 50**, or 100 mg/kg bw/day Procymidone: 5, 10, **25, 50, 100** or 150 mg/kg bw/day DEHP: 3, 10, 30, or **100** mg/kg bw/day	16(15) 8(5-8) 8(5-8) 16/8(6-8/14)
(4) Mixture study of vinclozolin, flutamide and procymidone Mixture ratio 31:1:18 **(Paper II, III, IV)**	**Control**: vehicle-dosed Mixture: **7.87, 19.67**, 39.3, 70.8, or 106.2 mg/kg bw/day Vinclozolin: **24.5**[b] or 95.9 mg/kg bw/day Flutamide: 0.77[b] or 3.86 mg/kg bw/day Procymidone: 14.1[b] or 61.8 mg/kg bw/day	16(14) 16(7/12-15) 16(12-14) 8(6-7) 8(5-6)
(5) Mixture study of vinclozolin, finasteride, DEHP and prochloraz Mixture ratio 500:1:300:500 **(Paper V)**	**Control**: vehicle-dosed Mixture: **13.01, 65.05** or **130.1** mg/kg bw/day Vinclozolin: 5[c] or 50[e] mg/kg bw/day Finasteride: 0.01[d] or 0.1[e] mg/kg bw/day DEHP: 3[c], 15[d] or 30[e] mg/kg bw/day Prochloraz: 5[c], 25[d] or 50[e] mg/kg bw/day	16 (13) 16 (11-16) 8 (7-8) 8 (6) 8 (5-7) 8 (6-7)

Table 1. Overview of the studies in this PhD thesis.

* The number of pregnant rats often differed from chemical to chemical, that is the reason for the intervals. The studies were performed using 3-4 blocks (with one week in between), and all dose groups were equally represented in the blocks.

[a] This dose of finasteride induced perinatal death and low pup weight and had to be decreased to 50 mg/kg bw/day from PND1-3 (block 1) and from GD 18 (block 2), from GD 11 (block 3), and in block 4 the dams received 50 mg/kg bw/day during the whole dosing period. 50 mg/kg bw/day was used for the data analysis. [b] Dose of the single chemical included in the 39.3 mg/kg bw/day mixture dose Study 4. [c] Dose of single chemical present in the 13.01 mg/kg bw/day mixture dose. [d] Dose of the single chemical included in the 65.05 mg/kg bw/day mixture dose. [e] Dose of the single chemical included in the 130.1 mg/kg bw/day mixture dose.

The dose groups **in bold** are included in the behavioural tests, but in MIX 130.1 (study 5) only the females were included.

An overview of the postnatal test battery included in this thesis is given in Table 2.
Typically 1 male and 1 female from each litter were weaned on PND 21, and kept for later behavioural testing and for examination of external malformation at PND 47. The weaned offspring was housed in pairs of the same sex and exposure status. In some cases two males or two females were kept from the same litter to be able to house the animals in pairs.

End point / function	**Test**	**Age at testing**	**Included in study**
Physical development	Anogenital distance (AGD)	PND 0	All studies
	Nipples (NR)	PND 12±1	All studies
	Dysgenesis (male)	PND 16	All studies
	Malformations (male)	PND 47	Study 4 & 5 (mixture)
Play behaviour	Rough and tumble play	PND 30	Study 2, 3 & 4
Motor activity	Activity	Young adults 8-9 weeks	Study 2
	Habituation	Adults 16-20 weeks	Study 2-5
Spatial learning and memory	Morris maze, learning	8-12 weeks	Study 2-5
	Morris maze, memory	14-16 weeks	Study 2-5
	Morris maze, reversal and new learning	14-16 weeks	Study 2-5
Sweet preference	Two bottle test	19 -23 weeks	Study 3-5
Sexual behaviour	Mating behaviour with exposed male and female rat	25-30 weeks	Study 4 & 5 (mixture)

Table 2. Postnatal test battery in the studies.

5. Results from this project

This thesis deals primarily with investigations of the effects of anti-androgenic substances on the development of the male rat with special focus on AGD, NR, external malformations and sexual dimorphic behavioural effects.

The thesis is based upon the five papers comprised in part two and the behavioural data presented in appendix 1 part two. Below, the aims, results and conclusions of the investigations in these five papers will briefly be presented followed by a short presentation of aims, results and conclusions of the behavioural studies. The applied methods, study design, and test compounds are summarised in Tables 1 and 2.

5.1 Paper I

The aim of paper I was to study the whole dose-response curve for the demasculinising effects of DEHP in rats as well as investigating low dose effects. Our results from two studies (study 2 and 3) demonstrate that DEHP at a relatively low dose of 10 mg/kg bw/day causes adverse anti-androgenic effects on male rat development. At this dose level, male AGD distance was decreased, the incidence of NR was increased, the weight of the levator ani/bulbocavernosus muscle was reduced, and mild external genitalia dysgenesis/malformation was observed. Moreover, higher doses of DEHP, i.e. from 100 mg/kg bw/day, additionally induced histopathological effects on the testes, reduced testicular and prostate weights, and reduced expression of androgen-regulated genes: PBP C3 (Prostate binding protein subunit C3) and ODC (ornithine decarboxylase) mRNA in the prostate. The results provide new evidence of low-dose effects of DEHP, supporting that the NOAEL of 5 mg/kg bw/day for DEHP that has been established in the EU is appropriate (REF. EU RAR).

Based on the results from these studies with DEHP, we selected 3 mg/kg bw/day as our NOAEL value in the mixture study with 4 dissimilar acting anti-androgens (presented in Paper V).

5.2 Paper II

The aims of paper II were to assess whether the combined effects of three androgen receptor antagonists (vinclozolin, flutamide and procymidone) could be predicted based on dose-response data of the individual chemicals by employing the concept of dose addition (Loewe and Muischnek 1926), and to investigate the ability of low mixture doses to induce disruption of male sexual differentiation after *in utero* and postnatal exposure. Vinclozolin, flutamide and procymidone were combined at a mixture ratio (31:1:18) proportional to their individual potencies for causing

retention of 6 nipples in male offspring. These individual potencies were based on extensive dose-response studies with the individual mixture components.

Focusing on AGD and NR in male rats, the results revealed that the combined effects of the three anti-androgens were dose-additive for AGD and that the observed responses for NR were slightly higher than those predicted on the basis of dose addition.

A combination of doses (MIX 19.7) of each chemical, which on their own did not change AGD statistically significantly, induced clear combination effects. Furthermore, exposure to low doses of the individual chemicals showed only modest effects on NR, while the mixture (MIX 7.9) induced NR in the males that clearly approached female values.

In conclusion, effects of a mixture of similarly acting anti-androgens can be predicted fairly accurately based on the potency of the individual mixture components by using dose addition. Exposure to anti-androgens, which individually appears to exert only small effects, may induce marked responses, in combination with possibly unrecognized, similarly acting chemicals.

5.3 Paper III

The paper describes further results from the mixture study of the 3 AR receptor antagonists (vinclozolin, flutamide and procymidone).

This paper focuses on effects at the morphological and molecular level, e.g. changes of reproductive organ weights and histopathology, malformations of male external genitals, and gene expression levels in prostates from male rat pups were chosen as endpoints for extensive dose-response studies. The aim of paper III was to elucidate if the combined effects of the three anti-androgens (vinclozolin, flutamide and procymidone) act as dose-additive compounds at these endpoints as well, and to determine whether small effects when judged on a chemical-by-chemical approach, induces marked responses in combination as previously seen for AGD and NR (Paper II). With all reproductive organs weights and expression of PBP C3 mRNA in prostates as the endpoints, the combined effects of the three anti-androgens were dose-additive. Histological evaluation showed that dysgenesis/malformation and/or hypoplasia of prostates, seminal vesicles, and epididymis was seen with the highest doses of the mixture (MIX 106.2). No changes were observed in any of the single-compound low-dose groups for any of these lesions. Dysgenesis/malformation of external genitalia was observed with all doses of the mixture and severe dysgenesis/malformation was seen only with a mixture for which the individual compounds did not cause any such effects (MIX 39.3).

In conclusion, the results from paper III support the findings of paper II, that combination of similarly acting anti-androgens are able to affect the male rat offspring. These effects can be

predicted fairly accurately on the basis of information about the potency of the individual mixture components by using the concept of dose addition. There are clear indications that anti-androgens act together to produce marked combination effects when combined at doses that individually produce statistically insignificant responses.

5.4 Paper IV

The paper describes further results from the mixture study of the 3 AR receptor antagonists (vinclozolin, flutamide and procymidone).

The aim of paper IV was to assess the frequencies of hypospadias and other external sexual malformations in the young adult male rats exposed pre- and postnatally to vinclozolin, flutamide or procymidone, or a mixture of these (as described in paper II and III). An additional aim was to examine if the anti-androgenic effects observed in the male pups, i.e. AGD, NR and malformations/dysgenesis PND 16 were predictive early biomarkers of external malformations observed in the sexually mature male rats later in life. Markedly increased frequencies (56%) of clear external malformations (hypospadias) were observed after exposure to a mixture of the three chemicals compared to administration of the three chemicals alone (0%). AGD at PND 1, NR at PND 13, and dysgenesis score at PND 16 were highly correlated with the occurrence of hypospadias, and combination effects were seen at doses where each of the individual chemicals caused no observable effects. AGD was a good early biomarker, as a 25% reduction in mean AGD measured on postnatal day 1 is likely to result in clear malformations in approximately 50% and marked malformations in approximately 25% of the adult male rats. Already at approximately 8% reduction in mean AGD, the likelihood for slight and clear malformations was increased.

In conclusion the results from this paper indicate that doses of anti-androgens, which appeared to induce no hypospadias when judged on their own, might induce a very high frequency of hypospadias when they interact in combination with other anti-androgens. Moreover, changes in the early biomarkers ‘AGD, NR and dysgenesis at PND 16’ could predict malformations in the adult male rat.

5.5 Paper V

Our second mixture study focused on the effects of four dissimilarly acting anti-androgens, i.e. vinclozolin (androgen receptor antagonist), DEHP (testosterone synthesis inhibitor), finasteride (inhibitor of steroid type II 5α-reductase) and prochloraz (multiple mechanisms incl. androgen receptor antagonism and decreased testosterone levels). The 4 chemicals were combined at their

respective NOAELs (MIX 13.01), 5 times the NOAELs (MIX 65.05) and 10 times the NOAELs (MIX 130.1) based on our dose-response studies. AGD, NR, and reproductive organ weights at PND 16 were clearly affected in the mixture groups at dose levels where the individual chemicals alone caused no or only minor effects. The combined effects were equally well predicted by dose-addition or independent action. Alterations in retained nipples were the most sensitive endpoint, with effects becoming noticeable at the lowest doses. Changes in AGD were almost as sensitive, followed by reductions in prostate and LABC weights, and genital malformations. Finasteride was by far the most potent chemical. It exhibited dose-response relationships with very shallow gradients for all endpoints. In addition, a very high frequency of external malformations (PND16 and 47) such as hypospadias was seen for a mixture dose (MIX 130.1) at which the individual compounds caused no significant effects on external malformations. At the dose of vinclozolin included in this mixture (50 mg/kg bw/day), a low non-significantly increased frequency was observed with 4.3% malformations at PND 16 and 12.5% malformations at PND 47.
The results indicate that dose-addition models may predict the combined effects of anti-androgens accurately and that marked effects can occur at doses below NOAELs for the single chemicals. Moreover, the experimentally observed responses on malformations exceeded the predictions, suggesting that the combined effect of DEHP, vinclozolin, prochloraz and finasteride is synergistic with respect to genital malformations.

A summary of the above mentioned results are presented in table 3a (first mixture) and 3b (second mixture) to give an overview of the findings in paper I-V.

Table 3a. Summary of effects from the first mixture study in male rat pups exposed to flutamide (FLU), vinclozolin (VIN), procymidone (PRO), or a mixture of flutamide, vinclozolin, and procymidone (MIX) from GD 7 to PND 16. The results are based on paper II, III and VI. All statistically significant results, compared to controls, are shown as a "+" while the non-significant are shown as "-".

Endpoint	**FLU 0.77 mg/kg**	**VIN 24.5 mg/kg**	**PRO 14.1 mg/kg**	**MIX 39.3 mg/kg**	**Dose-additivity?**	**Joint effect compared to effect of single chemicals**
AGD index, PND 1	-	-	-	+	Yes	Marked joint effect; no significant effect of single chemicals
Nipple retention PND 13	+	+	+	+	Yes (slight synergy at high doses)	Marked joint effect; small significant effect of single chemicals
Right testis, PND 16	-	-	-	-	n.a	No joint effect; no significant effect of single chemicals
Epididymides, PND 16	+	+	+	+	n.a	Marked joint effect; significant effect of single chemicals
Ventral prostate, PND 16	-	+	+	+	Yes	Marked joint effect; no or small significant effect of single chemicals
Seminal vesicles, PND 16	-	-	-	+	Yes	Marked joint effect; no significant effect of single chemicals
LABC[#], PND 16	-	+	-	+	Yes	Marked joint effect; no or small significant effect of single chemicals
Bulbourethral glands PND 16	-	+	-	+	n.a	Marked joint effect; no significant effects of FLU and PRO, but clear significant effect of VIN
PBP C3 expression in prostate, PND 16	-	-	-	+	Yes	Marked joint effect; no significant effect of single chemicals
Dysgenesis of external reproductive organs, PND 16	-	-	-	+	n.a	Marked joint effect; small significant effect of single chemicals
Malformation of external reproductive organs, PND 47	-	-	-	+	n.a	Marked joint effect; no significant effect of single chemicals

[#] Levator ani/bulbocavernosus muscles, n.a.: not analysed

Table 3b. Summary of effects from the second mixture study in male rat pups exposed to vinclozolin (VIN), finasteride (FIN), Di-(2-ethylhexyl) phthalate (DEHP) and prochloraz (PZ), or mixtures of VIN, FIN, DEHP and PZ from GD 7 to PND 16. The results are based on paper V. All statistically significant results, compared to controls, are shown as a "+" while the non-significant are shown as "-"

Endpoint	**VIN**	**FIN**	**DEHP**	**PZ**	**MIX**	**Dose-additivity?**	**Joint effect compared to effect of single chemicals**
AGD index, PND 1, NOAEL doses	-	-	-	-	+	Yes	Joint effect; no significant effect of single chemicals
Nipple retention, PND 13, number, NOAEL doses	-	+	-	-	+	Yes	Joint effect similar to effects of finasteride
Right testis, PND 16, % of control, all doses	-	-	-	-	-	n.a.	No joint effect; no significant effect of single chemicals
Epididymides, PND 16, % of control, 10 times NOAEL doses	+	+	-	-	+	n.a.	Marked joint effect; significant effect of single chemicals
Ventral prostate, PND 16, % of control, 10 times NOAEL doses	-	-	-	-	+	Yes	Marked joint effect; no significant effect of single chemicals
Seminal vesicles, PND 16, % of control, 10 times NOAEL doses	-	-	-	-	+	Yes	Marked joint effect; no significant effect of single chemicals
LABC[#], PND 16, % of control, 10 times NOAEL doses	-	-	-	-	+	Yes	Marked joint effect; no significant effect of single chemicals
Bulbourethral glands, PND 16, % of control, 10 times NOAEL doses	+	-	-	-	+	n.a.	Marked joint effect; similar to effect of vinclozolin
Dysgenesis of external reproductive organs, PND 16, 10 times NOAEL doses	-	-	-	-	+	Synergy	Marked joint effect; no significant effect of single chemicals
Malformations of external reproductive organs, PND 47, 10 times NOAEL doses	-	-	-	-	+	n.a	Marked joint effect; no significant effect of single chemicals

[#] Levator ani/bulbocavernosus muscles, n.a.: not analysed

5.6 Results from Behavioural studies (appendix 1)

The results from behavioural studies are presented more in details in appendix 1 and a summary is provided in Table 4.

Chemicals	**Development of reproductive external organs (LOAEL)**	**Behavioural (LOAEL)**	**Most sensitive endpoint**
Finasteride (FIN)	At FIN **0.01** mg/kg bw/day effect on NR, while effects on AGD and malformations were seen at 0.1 and 1 mg/kg bw/day, respectively	**FIN 10** mg/kg bw/day decreased activity in females	Nipple retention (NR)
Prochloraz (PZ)	**PZ 50** mg/kg bw/day for NR and >100 mg/kg bw/day for AGD	No behavioural effects in relation to PZ 10, 25 and 50 mg/kg bw/day	NR
Procymidone (PRO)	**PRO 14.1** mg/kg bw/day for NR and **25** mg/kg bw/day for AGD	**PRO 100** mg/kg bw/day females increased play behaviour	Both NR and AGD were more sensitive endpoints than behavioural effects
DEHP	**10** mg/kg bw/day for both NR, AGD	No behavioural effects in relation to DEHP 100 mg/kg bw/day	Both NR and AGD were more sensitive endpoints than behavioural effects
Flutamide	**0.5** mg/kg bw/day for NR	Flutamide exposed rats were not included in behavioural tests	NR; behavioural effects were not studied
Vinclozolin (VIN)	**5** mg/kg bw/day for NR and 10 mg/kg bw/day for AGD	**VIN 24.5 mg/kg bw/day** -females were more active -males swam shorter in the learning period - males mounted significantly more	NR
MIX 3 AR antagonists (Vinclozolin, Flutamide, Procymidone)	**MIX 7.9** mg/kg bw/day for NR and weight of epididymides **MIX 19.7** mg/kg bw/day also for ventral prostate and bulbouretral glands **MIX 39.3** mg/kg bw/day for effect on AGD	**MIX 7.9** and **MIX 19.7** learning impaired in males in Morris water maze	Behaviour was just as sensitive as the effects on the reproductive organs
MIX 4 dissimilar acting anti-androgens (Finasteride, Prochloraz, Vinclozolin, DEHP)	**MIX 13.01** mg/kg bw/day for effect on NR and AGD, while MIX 65.05 mg/kg bw/day showed malformations	**MIX 130.1** mg/kg bw/day females showed increased intake of sweet preference	Both AGD, NR and malformations were more sensitive endpoints than behavioural effects

Table 4 shows an overview of the LOAELs (Lowest adverse effect level) in this thesis and an indication which endpoint was the most sensitive in relation to a specific chemical or a mixture of chemicals.

Primarily, the aim of including behavioural testing was to study the sensitivity of sexually influenced behaviour compared to the development of reproductive external organs. Another reason for including these behavioural tests was to observe if the sexual difference in these tests had changed after exposure pre- and postnatally to anti-androgens.
It is expected that anti-androgens that lower the testosterone levels and/or inhibits the aromatase activity will contribute to a decreased conversion of testosterone into oestradiol, and thereby lower the oestradiol levels needed for complete behavioural masculinisation of the male rat brain. Another expectation could be that receptor mediated anti-androgens may block the androgen receptors in the brain, and thereby reduce the negative feedback that the endogenous testosterone exerts on the hypothalamus and pituitary, which will result in increased LH and testosterone production and thereby an increased masculinisation of the developing male rat.

5.6.1 Finasteride Effects on both motor activity, and learning and memory were found after exposure to finasteride. The decreased activity observed in females in two of the finasteride exposed groups (0.1 or 10 mg/kg bw/day) indicates behavioural changes in the male direction.
Behaviour was not the most sensitive endpoint regarding pre- and postnatal exposure to finasteride, as the male rats showed NR at a 1000 times smaller dose, and AGD was reduced at a 100 times smaller dose.

5.6.2 Prochloraz No significant effects of exposure to prochloraz (10, 25 and 50 mg/kg bw/day) were found on any of the behavioural endpoints tested. Furthermore, no significant effects of exposure were seen in the mixture study with four dissimilarly acting anti-androgens, where prochloraz was present at doses of 5 and 25 mg/kg bw/day.
Behaviour was not the most sensitive endpoint with regard to pre- and postnatal exposure to prochloraz, as the male rats showed NR at 50 mg/kg bw/day whereas we did not observe any behavioural effects at this exposure level.

5.6.3 Procymidone Increased play behaviour was found in females exposed to PRO 100 mg/kg bw/day compared to the control females. This indicates a masculinising effect of procymidone in the females. In the Morris water maze, PRO 50 mg/kg bw/day females (memory day 2) performed better (i.e. masculine direction), but no dose response relationship was found, as no consistent effect on memory of PRO 100 mg/kg bw/day females was observed. In the Morris water maze, PRO 25 mg/kg bw/day and PRO 50 mg/kg bw/day males (new learning) performed better (i.e. masculine

direction). However, in contrast to this, PRO 100 mg/kg bw/day males showed longer latency (i.e. feminised direction) so no consistent effects or direct dose-response relationship were found.

Behaviour was not the most sensitive endpoint when evaluating pre- and postnatal exposure to procymidone, as the male rats showed NR at an almost 10 times lower dose level and reduced AGD at a 4 times lower dose level than the dose level where the behaviour was affected (increased play behaviour in females, impaired learning in males).

5.6.4 Vinclozolin Increased activity was observed during the initial part of the period in females exposed to VIN 24.5 compared to control females.

In the Morris water maze, the VIN 24.5 males swam significantly shorter than control males and in mating behaviour, a significantly increased number of mounts were seen in VIN 24.5 exposed males.

Behaviour was not the most sensitive endpoint regarding pre- and postnatal exposure to vinclozolin, as the male rats showed NR at 5 mg/kg bw/day and reduced AGD from 10 mg/kg bw/day. The study only included the dose VIN 24.5 mg/kg bw/day where behavioural effects were observed (Table 4). The females' behaviour pointed in the direction of a feminised effect (more active) while the males' behaviour pointed in the direction of a masculinising effect (increased learning, increased mounting).

5.6.5 First mixture of 3 AR antagonists (vinclozolin, flutamide and procymidone).

Increased activity was observed, during the initial part of the period in females exposed to MIX 19.7 compared to control females. The males exposed to MIX 7.9 were less active (i.e. masculinised), but this was only significant in the last period. Furthermore, no dose response relationship was observed, as no effects were observed on males exposed to MIX 19.7.

Learning was impaired (i.e. feminised effect) in males dosed with both MIX 7.9 and MIX 19.7 when tested in the Morris water maze.

The behavioural testing showed that male behaviour could be just as sensitive an endpoint as the effects on reproductive organs after exposure to mixtures of 3 AR antagonists (vinclozolin, flutamide, and procymidone). The learning in Morris water maze was impaired at the same dose levels where effects on nipples, weight of epidydimides, ventral prostate and bulbouretral glands were affected.

5.6.6 Mix study of 4 dissimilarly acting anti-androgens (vinclozolin, finasteride, DEHP and prochloraz). The only effect on behaviour observed in this study was that the MIX 130.1 females showed an increased intake of sweet water compared to control females. This could be evaluated as increased feminisation in this group. Behaviour was not the most sensitive endpoint regarding pre- and postnatal exposure to a mixture of 4 dissimilarly acting anti-androgens, as the male rats showed NR and reduced AGD at a 10 times lower dose level (MIX 13.01 = Mixture of NOAELs) than where the effects on female behaviour were seen (MIX 130.1 effects on sweet preference in females and severe malformations in males).

6. Discussion

6.1 Effects of single chemicals

Dose-response analysis of all individual mixture components (flutamide, procymidone, DEHP, vinclozolin, prochloraz and finasteride) were carried out to provide a basis for the calculation of the expected additive effects for mixtures of specific compositions. The dose-response studies for the single chemicals were used to design two large developmental toxicity mixture studies and to predict the effects of a combination with constant mixture ratios. The dose response studies were performed also to define the NOAELs of the different chemicals. The compounds were given individually (by gavage) to pregnant rats throughout gestation and lactation, covering the period when male sexual differentiation takes place.

The observed NOAEL values for nipple retention (NR), the most sensitive endpoint, from these dose response studies are general in accordance with regulatory NOAELs as seen in Table 5.

Compound	Our NOAEL (NR) mg/kg bw/day	Regulatory NOAEL mg/kg bw/day	Comments	References
DEHP	3	5	Based on effects on testes	(EU RAR 2004)
		Between 1 and 10	Based on effects on Leydig cells	(Kavlock et al. 2006)
Vinclozolin	5	4	Study of reproductive toxicity	RAR from http://www.inchem.org
		4.9	2. Generation study in rats	BASF Study http://www.inchem.org
Flutamide	0.5	0.6 *		(Miyata et al. 2002)
Procymidone	10	12.5	2. Generation study in rats	RAR from http://www.inchem.org/documents/jmpr/jmpmono/v89pr12.htm
Prochloraz	5	3.7	Multigenerational reproductive toxicity in rats	JMPR 2001 http://www.inchem.org/documents/jmpr/jmpmono/2001pr11.htm
Finasteride	0.01	0.10	Based on hypospadias	MERCK Propecia® (Finasteride) Prescribing information http://www.merck.com/product/usa/pi_circulars/p/proscar.html
		0.03	Based on NR	
		0.003	Based on AGD	

Table 5. Comparison of NOAELs estimated for nipple retention (NR) in the present studies with those currently used for regulatory purposes in human risk assessment * No regulatory NOAEL value was found for Flutamide and the 0.6 value is reported in a research paper (Miyata et al. 2002).

6.2 Effects of combined exposure to similarly acting anti-androgens

Our study has shown clearly that antiandrogens (vinclozolin, flutamide, and procymidone) with a similar mode of action (androgen receptor antagonism) act together in an additive way and the results could, as expected, be predicted based on dose addition for a broad spectrum of endpoints assessed in rat pups before weaning (Hass et al. 2007; Metzdorff et al. 2007).

These findings are consistent with the previously reported additivity of antiandrogens on other endpoints such as AR receptor activation *in vitro* and *in vivo* (Nellemann et al. 2003; Birkhøj et al. 2004). Also, a combination of doses of each chemical that, on its own did not produce significant effects, induced marked combination effects on AGD, NR, reproductive organ weights, and PBP C3 gene expression in the prostate, and caused marked dysgenesis of external reproductive organs on PND 16. This phenomenon, somewhat provocatively dubbed "something from 'nothing'", has been observed with multi-component mixtures of oestrogenic agents in reporter-based assays (Silva et al. 2002; Rajapakse et al. 2002), the Uterotrophic assay (Tinwell and Ashby 2004), and vitellogenin induction in fish (Brian et al. 2005).

The results in paper IV indicate that the "something from 'nothing'" phenomenon may also apply to hypospadias in young adult male rats exposed to similarly acting anti-androgens during development. In this case, a combination of 24.5 mg/kg bw/day vinclozolin, 0.77 mg/kg bw/day flutamide and 14.1 mg/kg bw/day procymidone induced hypospadias in almost 60% of the male offspring, whereas the incidence after exposure to 24.5 mg/kg bw/day vinclozolin alone was similar to the untreated controls, i.e. 0%. This suggests that the presence of flutamide and procymidone in the mixture gravely exacerbated the effects of vinclozolin. Taken together, these clear correlations demonstrate that the early endpoints in pups (AGD, NR and dysgenesis in the males at PND 16) are good predictors of the malformations observable later in adult/sexually mature male rats.

The findings from the mixture study with similarly acting anti-androgens clearly indicate that risk assessment based on NOAELs for single anti-androgens alone underestimate the risk for hypospadias and other adverse anti-androgenic effects.

6.3 Effects of combined exposure to dissimilarly acting anti-androgens

The second mixture study focused on the effects of four dissimilarly acting anti-androgens. The combined effects were equally well predicted by dose-addition or independent action for AGD, NR, and weight changes of the prostate and LABC. That this occurred despite the different mechanistic premises that underlie the two prediction concepts is coincidental (paper V).

When the four chemicals were combined at doses equal to no-observed-adverse-effect levels estimated for nipple retention (NR NOAEL), significant reductions in AGD were observed in the male offspring. In addition, a very high incidence of external malformations such as hypospadias was seen for a mixture for which the individual compound caused no significant effects on malformation incidences. This combination effect was synergistic and worse than predicted based on dose-addition or independent action ((Christiansen et al. 2009)submitted, paper V). The male rats showed NR and reduced AGD at a 10 times lower dose level (MIX 13.01 = Mixture of NOAELs) than the dose level where the malformations in nearly all male offspring were seen.

The significance of these findings in rats for human risk assessment must be emphasised, because they clearly indicate that assessing the potential risk of anti-androgens on a chemical-by-chemical approach may severely underestimate the real human risk for hypospadias and other adverse anti-androgenic effects.

In conclusion, our findings challenge the widely held view (COT 2002; VKM 2008) that chemical mixtures exhibiting various modes of action are without combination effects at levels near supposed dose thresholds, used in regulatory toxicology as points of departure for the derivation of tolerable human exposures. If this assumption is correct, we should not have been able to observe alterations in AGD when all four chemicals were combined at their NOAELs (based on nipple retention).

Our findings echo the observations made by Cynthia Rider and colleagues. They examined a mixture of 7 dissimilarly acting anti-androgens in rats (butyl benzyl phthalate, di-*n*-butyl phthalate, DEHP, vinclozolin, procymidone, linuron, and prochloraz). They found that this mixture disrupted the male rat reproductive tract differentiation and induced malformations that exceeded those predicted by dose addition (Rider et al. 2008). Because their data for the single chemicals were sometimes based on dosing regimens that differed from those used in the mixture experiment, there is a degree of uncertainty as to whether the excess malformations in that study represent a true synergism. Our evaluations are based on dose-response data for single chemicals and mixtures from one large study and therefore substantiate concerns about the synergistic induction of genital malformations by mixed-mode of action anti-androgens.

Recent epidemiological studies suggest an association between an increased risk of hypospadias and maternal occupational exposure to phthalates and hair sprays (Ormond et al. 2009).

Recent studies suggest a previously unidentified role for the progesterone receptor, possibly interacting with the androgen receptor, in disturbed genital tubercle development (Willingham et al. 2006). *In utero* exposure to natural or synthetic progesterones can increase the risk of hypospadias

in male mice. Intake of natural or synthetic progesterone during pregnancy in humans has been associated with an increased risk of hypospadias (Carmichael et al. 2005; Willingham et al. 2006). Prochloraz, one of the chemicals in the mixture, was able to induce increased testicular progesterone concentrations in male rat foetuses, and this effect occurred at lower dose levels than those present in the mixture investigated here (Vinggaard et al. 2005; Laier et al. 2006; Blystone et al. 2007). Thus, elevated progesterone levels due to prochloraz exposure may have a role in the synergisms with severe hypospadias that we observed in our study Paper V. The results from our study with dissimilar mixtures support what is concluded in Kortenkamp et al. (2007), stating that "the view that mixtures of dissimilarly acting chemicals are "safe" at doses below NOAEL does not only lack empirical support, it is also based on the erroneous assumption that NOAELs are indeed zero effect levels" (Kortenkamp et al. 2007).

6.4 Behavioural effects and sensitivity

In conclusion, it is at present difficult to predict what happens to the behaviour of male and female rats pre- and postnatally exposed to anti-androgenic compounds. In some cases, the effects observed point in the direction of a masculinising effect on the males and a feminising effect on the females, while the morphological anti-androgenic effects are demasculinised male organs.

The expectation in relation to behaviour of male rats exposed *in utero* to mixtures of AR antagonists is that the negative feedback from testosterone will be reduced, resulting in increased LH and testosterone production and thereby an increased masculinisation of the developing male rat.

In most of the studies in this thesis, the development of the male reproductive organs was a more sensitive endpoint than behaviour. Nevertheless, animal behaviour seemed to be just as sensitive as the development of reproductive organs when testing the mixtures of 3 AR antagonists. In the Morris water maze, the learning was impaired in the males from MIX 7.9 and MIX 19.7 (i.e. results are in the direction of a more female like behaviour and thereby incomplete masculinisation). This was an unexpected result and could also be a result possibly related to a neurotoxic effect. Thus, in future testing of chemical mixtures it will be very relevant to look also at behavioural effects as these results could contribute to a broader picture of the toxicity of mixtures of EDCs that only takes the development of reproductive organs into account.

6.5 Input for existing OECD guidelines

As AGD and NR in these studies as well as many other studies are endocrine sensitive endpoint it is considered relevant to include assessment of these endpoints in both F1 and F2 offspring as a

standard parameter in the 2-generation study without the need for triggering this by alterations in sex ratio or timing of sexual maturation. In addition, the parameters could be included in the one-generation study and AGD could be included in the reproductive toxicity screening tests, i.e. TG 421 and TG 422. As mentioned in the background section (2.0) these endpoints are planned to be included in the new OECD guideline for the extended One-generation study. Malformations of external sex organs (e.g. hypospadia) in adult males are studied in only one male per litter in the existing two-generation study. This was also the case in the studies in this project, but recent power calculations presented at an OECD meeting in October 2008 show that the sensitivity can be markedly increased by looking at 2-3 males per litter (Hass, pers. com). This is discussed in relation to the new OECD guideline and recommended here, because hypospadia is a serious adverse effect caused by both single anti-androgens and their combinations in the studies in this thesis.

6.6 Future perspectives and input for mixture risk assessment

The research performed in this thesis aims to explore the need for improving the research based knowledge for future risk assessment approaches and also to give input to the regulatory system. Until now, the regulatory systems have no consistently accepted method for cumulative risk assessment for mixtures of chemicals.

A view that has persisted over the last 30 years is that combination effects do not occur when each chemical is present at doses equal to their NOAELs (COT 2002; VKM 2008). This view is challenged in the mixture study of dissimilarly acting anti-androgens((Christiansen et al. 2009), submitted, paper V). Alterations in AGD were observed when all four chemicals were combined at their NOAELs (based on nipple retention). It can, on that basis, be concluded that a risk assessment based on the NOAELs of single chemicals may underestimate the human risk for endocrine disrupting effects. It should be considered to take effects of mixtures into account in a risk assessment as a considerable part and perform the risk assessment for the anti-androgens in total regardless of their anti-androgenic mechanism.

Clearly it would never be realistic to test the potential effects of all possible combinations of chemicals with endocrine disrupting properties to which target groups of humans and/or wildlife could be exposed. Instead, it is necessary to develop robust predictive models that allow the effects of interactions of mixtures of chemicals with endocrine disrupting properties with common or different mechanisms of action to be evaluated. Data from the literature and this thesis suggest that the dose addition model adequately describes the effects of mixtures of endocrine disrupting chemicals with both similar and dissimilar modes of action (Kortenkamp 2007; Kortenkamp et al.

2007). The concept of dose addition could lead to a "mixture no-observed-adverse-effect level" (MNOAEL) for endpoints relevant to endocrine disruption. These could then be combined with an assessment factor to arrive at estimates of tolerable human exposure (EDEN 2007). The results from the studies in this thesis underline the importance of the ongoing initiatives in both EFSA and US Academy of science concerning the development of cumulative risk assessment.

6.7 Recommendations for future research

The results from the mixture studies will provide strong evidence in future research with mixture exposure of endocrine disrupting chemicals.

In EDEN the focus was on anti-androgens with similar or dissimilar mechanisms of action, but humans are exposed to a mixture of chemicals with different modes of action. The Department of Toxicology and Risk Assessment is a part of a new ongoing EU project called CONTAMED (2008-2011). This study will investigate the possible role of mixtures of 10-12 environmentally relevant EDCs (e.g. anti-androgens, oestrogens and, other classes of chemicals) in producing adverse developmental toxicity effects, including long-term effects, at dose levels at a mixture ratio proportional to human exposure, in a large extended developmental toxicity rat study. The results of these studies will be analysed jointly using dose-addition modelling, and they are expected to provide important information concerning applicability of dose-addition models for prediction of combined effects of EDCs and human safety evaluation of mixed exposure to EDCs.

In planning of experimental studies with simultaneous exposure to several chemicals, it is necessary with good dose-response data for the single chemicals. Moreover, it is important with thorough considerations concerning choice of dose, mixture of the chemicals and prediction model. It is highly recommended in the planning:

- to choose doses, which cover the whole dose response curves for these combination effects,
- to evaluate the chemicals according to potency
- to mimic the actual exposure of humans

7. Main conclusions

In vivo reproduction studies with rats indicate that dose-addition models could in most cases predict the combined effects of anti-androgens, and that marked effects can occur at mixture doses below NOAELs for the individual chemicals. The significance of these findings for human risk assessment must be emphasised, because they clearly indicate that risk assessment based on NOAELs for single anti-androgens alone may underestimate severely the risk for hypospadias and other adverse anti-androgenic effects.

Our results hold the promise that predictions of combination effects could be obtained in the future without conducting mixture experiments, by employing modelling approaches. Considering the high cost and long duration of reproductive toxicity studies this might greatly aid efforts in regulatory toxicology. Behavioural testing could in some cases provide useful complementary information and contribute to a broader picture of the toxicity of the mixtures of EDCs than studies that only take the development of reproductive organs into account.

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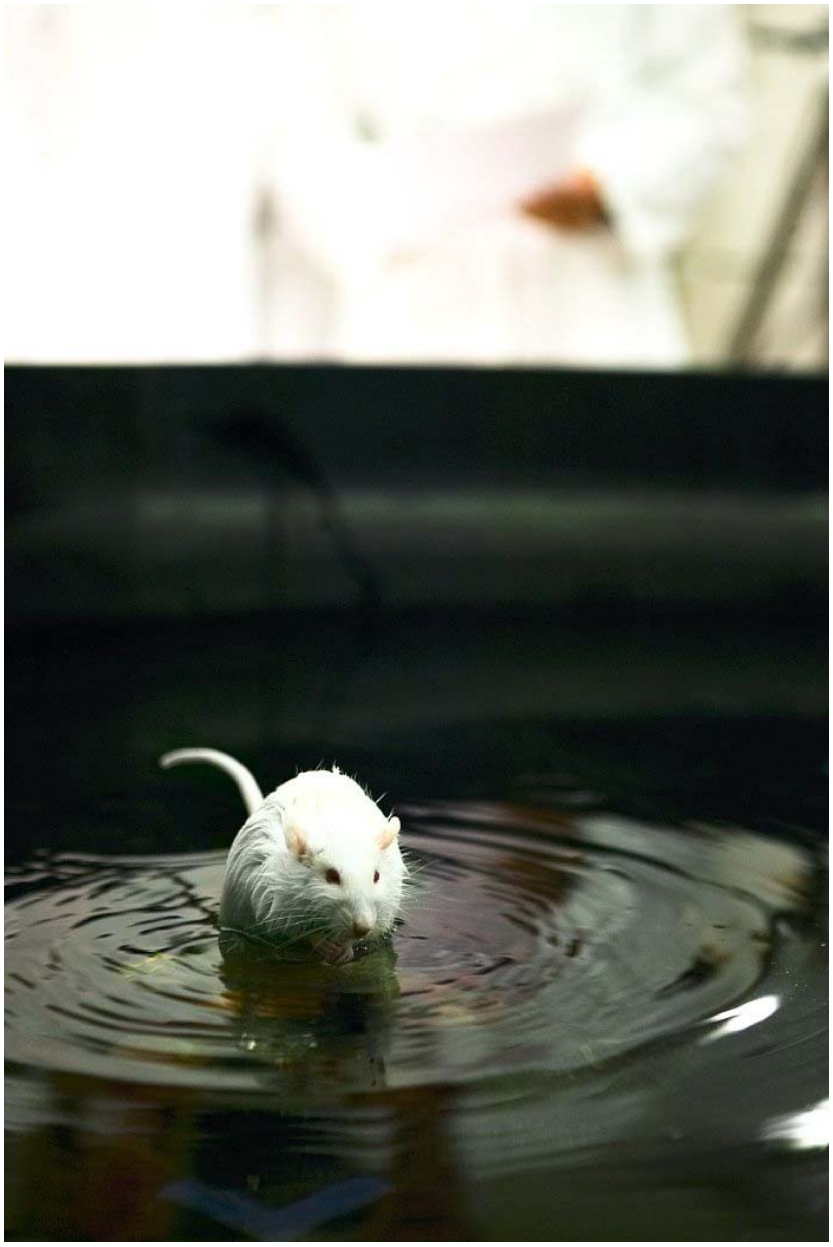
PART TWO

Appendix 1
Presenting behavioural studies included in the PhD thesis.



Introduction

During the 'critical periods' of development the central nervous system is very sensitive, and altered hormone levels as well as chemical exposure can have severe and permanent effects on development. This phenomenon may change the course of sexual differentiation. Consequently, a new test guideline for testing of potential developmentally neurotoxic chemicals has been developed, "OECD Test Guideline 426 for Developmental Neurotoxicity Study" (OECD 2007). This guideline study is designed to achieve data on the potential functional and morphological hazards to the nervous system, which may arise in the offspring following exposure of the mother during pregnancy and lactation. The evaluation of the offspring includes observations to detect gross neurological and behavioural abnormalities; to assess physical development, reflex ontogeny, motor activity, motor function, sensory function, and learning and memory; and to evaluate brain weights and neuropathology.

The following behavioural endpoints: play behaviour, activity, learning and memory (Morris water maze), sweet preference, and mating behaviour were included as a part of this PhD thesis. Primarily, the aim of including behaviour was to study the sensitivity of sexually influenced behaviour compared to the development of reproductive external organs. Another reason to include these behavioural tests was to observe if the differences between sexes in these tests had changed after exposure pre- and postnatally to anti-androgens.

It is well-known that there is a natural sex-difference in the reproductive behaviour of male and female rats. In addition, sex differences in a large number of other behavioural endpoints depend on early gonadal hormone secretion (MacLusky and Naftolin 1981). Sex differences in central nervous function represent the outcome of interactions between several different factors, among which the hormones secreted by the gonads during development are of principal importance. In mammals, the intrinsic behavioural pattern is generally female, with differentiation towards masculine pattern of behaviour occurring in the male as a result of exposure to testicular hormones during development. The conversion of testosterone into oestradiol by aromatase in the brain, however, is critical for the organization of the male brain in rats (Hotchkiss et al. 2002).

It has been shown in animal models that female rats show higher spontaneous activity than males and that they have a higher preference for sweetened water in a sweet preference test (Valenstein et al. 1967; MacLusky and Naftolin 1981). Gender differences for spatial learning abilities have been reported in both humans and rodents, where males generally perform better than females (Roof and Stein ; Beatty 1979; Williams and Meck 1991). The expected gender differences in the behavioural

tests included in this thesis are as follows: Play behaviour, males do more pinnings (Hotchkiss et al. 2002; Casto et al. 2003); motor activity, males are less active (MacLusky and Naftolin 1981); Morris water maze, males perform better than females (Williams and Meck 1991); and sweet preference test, females reported to consume more sweetened water in relation to body weight than males (Valenstein et al. 1967; Kaya et al. 2002).

It is expected that anti-androgens that lower the testosterone levels and/or inhibits the aromatase activity will contribute to a decreased conversion of testosterone into oestradiol, and thereby lower the oestradiol levels needed for complete behavioural masculinisation of the male rat brain. This will result in a demasculinisation. An expectation for the receptor mediated anti-androgens is that they block the androgen receptors in the brain, and thereby reduce the negative feedback that the endogenous testosterone exerts on the hypothalamus and pituitary, which will result in increased LH and testosterone production and thereby an increased masculinisation of the developing male rat.

Materials & methods

Study design

Behaviour was examined in offspring from study 2-5 and the different chemical exposures and dose groups are presented in Table 1. Time mated nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were supplied at day 3 (GD 3) of pregnancy. They were dosed by gavage from early gestation GD 7 until the day before expected birth, and then from PND 1 until PND 16. The studies were performed using 3-4 blocks (with one week in between), and all dose groups were equally represented in the blocks. Further details about the laboratory animal housing and studies are reported in Papers I-V.

Study (No.) Paper	**Groups and doses All dams exposed from GD 7-PND 16**	**No. mated per group (pregnant*)**
(2) Finasteride and DEHP, dose-response Procymidone range finding **(Paper I, V)**	**Control**: vehicle-dosed Finasteride: 0.001, 0.01, **0.1, 1, 10** or 100[a] mg/kg bw/day DEHP: 10, 30, 100, 300, 600 or 900 mg/kg bw/day Procymidone: 25 or 200 mg/kg bw/day	16 (15) 8/10 (7-9) 8(6-8) 4(2-3)
(3) Prochloraz, Procymidone and DEHP, dose-response **(Paper I, V)**	**Control**: vehicle-dosed Prochloraz: 5, **10, 25, 50**, or 100 mg/kg bw/day Procymidone: 5, 10, **25, 50, 100** or 150 mg/kg bw/day DEHP: 3, 10, 30, or **100** mg/kg bw/day	16(15) 8(5-8) 8(5-8) 16/8(6-8/14)
(4) Mixture study of vinclozolin, flutamide and procymidone Mixture ratio 31:1:18 **(Paper II, III, IV)**	**Control**: vehicle-dosed Mixture: **7.9, 19.7**, 39.3, 70.8, or 106.2 mg/kg bw/day Vinclozolin: **24.5**[b] or 95.9 mg/kg bw/day Flutamide: 0.77[b] or 3.86 mg/kg bw/day Procymidone: 14.1[b] or 61.8 mg/kg bw/day	16(14) 16(7/12-15) 16(12-14) 8(6-7) 8(5-6)
(5) Mixture study of vinclozolin, finasteride, DEHP and prochloraz Mixture ratio 5:0.01:3:5 **(Paper V)**	**Control**: vehicle-dosed Mixture: **13.01, 65.05** or **130.1** mg/kg bw/day Vinclozolin: 5[c] or 50[e] mg/kg bw/day Finasteride: 0.01[d] or 0.1[e] mg/kg bw/day DEHP: 3[c], 15[d] or 30[e] mg/kg bw/day Prochloraz: 5[c], 25[d] or 50[e] mg/kg bw/day	16 (13) 16 (11-16) 8 (7-8) 8 (6) 8 (5-7) 8 (6-7)

Table 1. Overview of the 4 studies that include behaviour in this PhD thesis. * The number of pregnant rats often differed from chemical to chemical, that is the reason for the intervals. The studies were performed using 3-4 blocks (with one week in between), and all dose groups were equally represented in the blocks.
[a] This dose of finasteride induced perinatal death and low pup weight and had to be decreased to 50 mg/kg bw/day from PND1-3 (block 1) and from GD 18 (block 2), from GD 11 (block 3), and in block 4 the dams received 50 mg/kg bw/day during the whole dosing period. 50 mg/kg bw/day was used for the data analysis. [b] Dose of the single chemical included in the 39.3 mg/kg bw/day mixture dose Study 4. [c] Dose of single chemical present in the 13.01 mg/kg bw/day mixture dose. [d] Dose of the single chemical included in the 65.05 mg/kg bw/day mixture dose. [e] Dose of the single chemical included in the 130.1 mg/kg bw/day mixture dose.
The dose groups **in bold** are included in the behavioural tests, but in MIX 130.1 (study 5) only the females were included.

Behavioural testing

All investigations of behaviour were performed between 9.00 a.m. and 4 p.m. during the animals' dark cycle, i.e. their active period. The behaviour was recorded by technicians who were blinded with respect to exposure groups. Exposed and control animals were tested alternately and so were females and males (except in the mating behaviour testing). Table 2 presents the test battery and describes the ages at which the testing was performed.

End point / function	Test	Age at testing	Included in study
Physical development	Anogenital distance	PND 0	All studies
	Nipples	PND 12±1	All studies
	Dysgenesis (male)	PND 16	All studies
	Malformations (male)	PND 47	Study 4 & 5 (mixture)
Play behaviour	Rough and tumble play	PND 30	Study 2, 3 & 4
Motor activity	Activity	Young adults 8-9 weeks	Study 2
	Habituation	Adults 16-20 weeks	Study 2-5
Spatial learning and memory	Morris maze, learning	8-12 weeks	Study 2-5
	Morris maze, memory	14-16 weeks	Study 2-5
	Morris maze, reversal and new learning	14-16 weeks	Study 2-5
Sweet preference	Two bottle test	19 -23 weeks	Study 3-5
Sexual behaviour	Mating behaviour with exposed male and female rat	25-30 weeks	Study 4 & 5 (mixture)

Table 2. Postnatal test battery in the studies.

Play behaviour

The play behaviour was observed, as two rats of the same sex and treatment were put together in the same cage for a period of 10 minutes. The endpoints scored for each pair of playing rats was the latency to the first pinning and the number of pinnings. A pinning was defined as either one of the rats standing over the opponent with its forepaws on the ventral surface of the other one. The play behaviour was recorded using a video camera with infrared light, making it possible to perform the test in an unlit room and without an observer present during testing (the same camera were used in the mating behavioural testing). The tested pairs of rats were separated for 48 hours before the observation, to increase their motivation for play, and the test was performed in their original "home cage" to make their environment as familiar as possible.

Motor activity and habituation capability

The testing of activity and habituation capacity has been described earlier by Axelstad et al (2008).

The animals were placed individually in clean plastic cages without bedding, and the cages were placed in activity boxes with photocells, which measured horizontal activity for 30 min (Fig. 1). The position of the cages in the activity boxes was adjusted in height, depending on the age of the rats. Neither food nor water was supplied during the measurement period. A computer in an adjoining room automatically recorded the output of the photocells and collected data for each of 10 three-minute intervals. The total activity during the 30 min observation period was used as a measure of general activity. In order to assess habituation, the 30 minutes were divided into two periods of 15 minutes.

Figure 1. The set up for the activity test, with the photocells shown at the right side (photo: Bo Herbst)

Learning and memory in Morris water maze.

The testing in Morris water maze has been described earlier by Hass et al., Hougaard et al. and Axelstad et al. and was carried out with minor modifications (Hass et al. 1995; Hougaard et al. 1999; Hass et al. 1999; Axelstad et al. 2008). The pool had a diameter of 220 cm, and a circular transparent platform was situated on a solid support and submerged 1 cm below the water surface, and thus invisible from water level. The animals were tested in four daily trials using four different starting points along the rim of the pool. When the rats reached and climbed onto the platform, the trial was completed. If the animal failed to locate the platform within 60 sec, it was led to the platform. A video-tracking device (Viewpoint video tracking system, Sandown Scientific, Middlesex, England) tracked the route of the animals, and the latencies to find the platform, the path lengths, and swimming speeds were used as endpoints.

The following scheme was used:

Learning. With the platform situated at the same place, the animals were trained in four trials per day for 5 consecutive days, until a stable performance was established.

Memory. Four weeks after the learning period, the animals were tested again with the platform still in the same quadrant of the pool. The animals were given four trials on each of 2 consecutive days.

New platform position (reversal learning and new learning). The day after the memory testing, the animals were tested in a reversal procedure (reversal learning) with the platform placed opposite to the original location. The following day, the platform was located in the centre of the maze (new learning). In both tests the animals were tested for four trials.

Figure 2. The set up for the sweet preference test, showing two bottles for each rat (photo: Bo Herbst)

Sweet preference test

The weeks before testing the animals were housed one per cage, and habituated to drinking from two bottles with tap water (Fig. 2). During the week of testing the animals were given a choice between tap water and water sweetened with 0.25% saccharin (Sigma-Aldrich). Position of the saccharin and water bottles were counterbalanced in each group to avoid position preferences. Each day the intake from both bottles was measured, and the bottles were refilled if needed. Body weights were registered in the same week and used for calculation of saccharin intake per 100 g body weight.

Mating behaviour

The male rats should be sexually experienced before the mating session with the exposed females as described by Chahoud and Faqi (Chahoud and Faqi 1998). For this purpose, non-ovariectomised sexually mature female Wistar rats (weight 170 g ± 20g) were purchased from Taconic Europe. These female rats were treated with β-oestradiol-3-benzoate (25 µg/rat) 48 hours before the mating and progesterone (500 µg/rat) 4 hours before mating to become in 'chemical oestrus'. Both solutions were injected subcutaneously. The effects of progesterone dosing last for about 4 hours and the females can be used several times for this purpose. The males were exposed to a female in 'chemical oestrus' for a maximum of 20 minutes. It was required that the female showed proceptive behaviour (described below) otherwise it was replaced by another female rat. The male rats had two

training trials in the test described below. If the male rat ejaculated before the 20 min, the training test was stopped otherwise the testing lasted for 20 minutes.

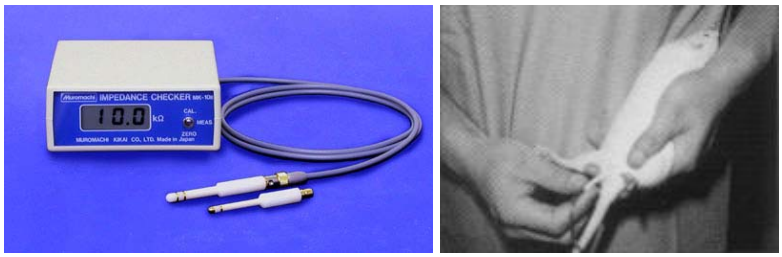


Figure 3. The Vaginal Impedance checker and measuring of impedance

Before each mating behaviour session with exposed female rats, a Rat Vaginal Impedance checker (MK-10C from Muromachi) was used. With this apparatus (Fig. 3) the electrical impedance of the epithelial cell layer of vaginal mucosa is measured at the frequency of 1 kHz by insertion of the probe into the vagina. In the pro-oestrus stage significantly higher impedance is produced compared to the other stages of the oestrus cycle. Behavioural oestrus corresponds to vaginal pro-oestrus, the 12-hour period before ovulation (Bartos 1977; Ramos et al. 2001).

In the morning between 9-11.30 am (in their active period), the impedance for the female rats was measured (Ramos et al. 2001). An impedance value at or above 3 kohm (kΩ, electrical impedance) indicated pro-oestrus and the female rat was selected for examining mating behaviour that day. The mating pairs consisted of a male and a female from the same exposure group and the person observing the mating behaviour was blinded with regard to exposure group.

One of the problems with this design of mating behaviour is that both males and females are exposed and as a result of this, it is unknown whether the observed behaviour is an effect of the female or the male exposure. This could also be the case in relation to play behaviour where both "players" are similar exposed.

The mating test was performed between 12 and 16 am (in the active period of the animals). The male from the couple was placed in a transparent polycarbonat cage (59.5 x 38 x 20cm (D x W x H)) with a flat lid and with no bedding in an unlit room. A female in prooestrus was then introduced into the male cage. The same cage was used for all animals in the study. The copulatory behaviour was recorded using a standard Phillips CCD-MOS video camera (black and white) with sensitivity in the infra- red area (800-950 nm). The camera was placed so that it could record all behaviour from the side of the cage. It was connected to a hard disk/DVD recorder (LVW-545 HDD+DVD recorder) and the recordings of mating behaviour were saved at this recorder. After 20 minutes the female was removed and vaginal smear taken.

All mating behaviour data were scored using a Psion Workabout (ProInfo) with the software Pocket Observer® (Noldus, The Netherlands) installed. This was done by trained observers blind to experimental groups until data processing in The Observer program was completed. The observer looked at the recordings of mating behaviour on a computer and registered the behavioural elements by pressing the following buttons on the Psion Workabout.

- K = Kick (female)
- V= Ear wiggling (female)
- L= Lordosis (female)
- M= Mount (male)
- I= Intromission (male)
- E= Ejaculation (male)

All of the elements are described below.

During behavioural oestrus or vaginal pro-oestrus, the female solicits the male to prompt him into mounting her. She darts towards him and runs or hops away. She may repeat this approach-retreat sequence several times, wiggling her ears. The male rat appears to finding these proceptive behaviours very attractive and if he mounts her, the pressure he exerts on her flanks, lower back, and anogenital area triggers lordosis, the female mating posture that enables copulation (Nelson 1995).

Kick: The normal female, when not in oestrus, will frequently kick the approaching male.
Ear wiggle: Female vibrates her ears rapidly. Ear wiggling is part of a suite of solicitation behaviours in which the female initiates and maintains mounting behaviour by the male. Ear wiggling occurs when the female is in behavioural oestrus, about every 4-5 days.
Lordosis: Is a female mating posture and is the females' response to the male mount. Female stands immobile, with her back arched downward toward the floor, her rump pushed upward and tail deflected to the side. Her vulva, which normally faces the floor, rotates almost 90 degrees to the vertical, backward-facing position. Without lordosis, copulation would be impossible. Lordosis is a reflexive behaviour that is triggered by a touch on the lower back, flanks, or genital region (Fig. 5).
Mount: One rat places its forequarters on another rat's rump from behind (Fig. 4). Mounting is the male copulatory position, and is seen when a male mounts a female prior to mating. Mounting is also sometimes seen between rats of the same sex, usually in an aggressive context.

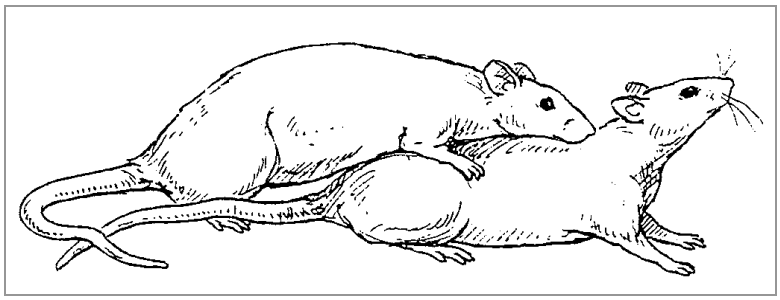


Figure 4. Mounting modified from (Hart 1969).

Intromission

Intromission is a sexual behaviour in which the male rat inserts his penis into the vagina during copulation. An intromission occurs during a mount, during which intra-vaginal penile insertion occurs and several high frequency thrusting movements occur (Fig. 5). After an intromission the male rat "jumps" away from the female rat.

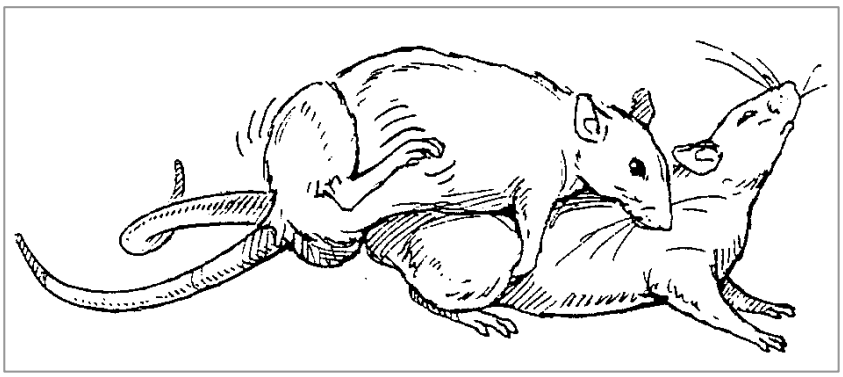


Figure 5.Intromission and lordosis modified from (Hart 1969).

Ejaculation

Ejaculation is the release of semen into the vagina. The thrusting is more prolonged during ejaculation, and the male often rises up behind the female post-ejaculation in a "freeze posture" in 2-3 seconds.

Statistical analysis

The litter was generally considered the statistical unit. When more than one pup per litter is investigated, two closely related statistical strategies are available to avoid inflation of sample size (Holson and Pearce 1992). The first alternative uses one score per litter, either a litter mean or the score of a single animal per litter. Second, one can include litter as an independent, random and nested factor in ANOVA (one way analysis of variance). The latter approach controls for litter effects and was generally applied for the statistical evaluation of behavioural data in these studies.

The two approaches are mathematically identical in their test for treatment effects (Holson and Pearce 1992).

The alpha level was set at 0.05 and tendencies reported were below 0.1. Data were examined for normal distribution and homogeneity of variance before using ANOVA in a mixed linear model ("Proc mixed nobound" in SAS).

In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall-Wallis test was used on one score per litter, followed by Wilcoxon's test for pair-wise comparisons.

The data from Morris maze (learning and memory) were analysed on a day by day basis for the endpoints length, latency and speed, thus not with repeated measures. If the overall analysis showed a significant gender difference in any of the endpoints in play behaviour, Morris water maze (leaning and memory) or the Sweet preference test, data from the two genders were also analysed separately. Pairwise comparisons were made with a Dunnett test.

Mating behaviour was analysed using a non parametric test (Fisher exact test).

Asterisks in figures, indicate a statistically significant difference compared to controls *: $p < 0.05$.

All analyses were done using SAS Enterprise Guide (version 3.0), SAS Institute Inc, Cary, NC, USA.

Results

Behavioural studies were not included in study 1 (vinclozolin and flutamide dose response study and prochloraz range finding study).

Study 2 Finasteride

In this study, offspring from the following groups were included in the behavioural studies: control, and finasteride (FIN): 0.1; 1 and 10 mg/kg bw/day. The number of animals was 12 males and 12-14 females from each group, representing 8 (FIN) and 12 (control) litters per group.

Play behaviour

No significant gender difference or effect of exposure was found.

Motor activity

In the young adults, no significant gender difference was found in the control group. Decreased activity in FIN 0.1 mg/kg bw/day and FIN 10 mg/kg bw/day was found in females during the last part of the testing period ($p<0.02$), but no significant effect on activity in females exposed to 1 mg/kg bw/day. Motor activity in young adults was not included in any of the later studies.
In the adult rats, no statistically significant gender difference was found in the control group. A statistically significantly decreased activity was seen in the group exposed to FIN 0.1 mg/kg bw/day ($p=0.008$), but only a tendency towards decreased activity was seen in FIN 10 mg/kg bw/day. This effect was most pronounced in females during the last part of the testing period (last 15 minutes). No significant effect on motor activity was found in the females exposed to FIN 1 mg/kg bw/day. These results showed decreased activity in the female rats exposed to FIN 0.1 and FIN 10 mg/kg bw/day at both ages tested. There is no clear dose-response relationship as no significant effects were seen in the intermediate group exposed to FIN 1 mg/kg bw/day. Consequently, the effect on motor activity observed in the lowest dose tested, FIN 0.1 mg/kg bw/day is questionable and may be a chance finding. However, the effect on female activity observed at the highest dose level of finasteride (FIN 10 mg/kg bw/day) is likely to be a true finding.

Morris water maze

In the 5-day learning period, no significant gender difference was found in the control group. In trial 5, a decreased latency to finding the platform was found in males exposed to FIN 0.1 mg/kg bw/day ($p=0.03$) and FIN 10 mg/kg bw/day ($p=0.04$), but no significant effect was observed in the

intermediate group exposed to FIN 1 mg/kg bw/day. No significant effects of exposure were observed in the females.

During memory and reversal learning, a significant gender difference ($p<0.02$) was found at day 1, day 2 and day , but no significant effect of exposure was found in relation to swim length, latency or swim speed in both males and females.

During the new learning testing period (platform located in the centre of the maze), a significant gender difference was found ($p=0.002$). Increased swim length ($p=0.03$) and latency to finding the platform ($p=0.006$) was found in males exposed to FIN 1 mg/kg bw/day compared to control males, but was not seen in the males exposed to FIN 10 mg/kg bw/day. In females, there were no significant effects of the exposure.

These results may indicate improved initial learning, but also an impairment of new learning ability in the finasteride exposed males. In both cases, however, there was no clear dose-response relationship.

Study 3 Prochloraz, procymidone and DEHP

In this study, offspring from the following groups were included in the behavioural studies: control, prochloraz (PZ) groups 10, 25 and 50 mg/kg bw/day; procymidone (PRO) groups: 25, 50 and 100 mg/kg bw/day; and DEHP group 100 mg/kg bw/day. The number of animals was 10-16 males and 10-16 females in each group representing 4-15 litters per group. Only statistically significant effects on the procymidone exposed rats is reported below as no significant behavioural effects of DEHP and prochloraz exposed rats were observed.

Play behaviour

No overall significant effect of group or gender was found in this test. The females exposed to PRO 100 mg/kg bw/day showed increased play behaviour compared to the control females ($p=0.003$ for pinnings, $p=0.004$ for latency to the first pinning, and $p=0.01$ for pinnings pr minute). These results indicate that the females exposed to PRO 100 mg/kg bw/day played significantly more compared to control females.

Activity adults

No significant gender difference or effects of exposure was found.

Morris water maze

In the learning period, no gender difference was found for any of the variables (swim length, latency or swim speed) and no significant effect of exposure was observed in males and females. On day 1 in the memory testing period, a significant gender difference was found (p=0.003), but there was no effect of exposure. On day 2, a significant gender difference was found (p=0.01). The females exposed to PRO 50 mg/kg bw/day showed shorter swim length (p=0.03) and shorter latency to finding the platform (p=0.02) than control females, but this was not seen in the PRO 100 mg/kg bw/day group. No significant effect of exposure was seen in the males. During reversal learning, no significant gender or exposure related differences were observed.

During new learning, a significant gender difference for the endpoint latency to finding the platform was found. The males in the groups exposed to PRO 25 and PRO 50 mg/kg bw/day showed shorter latency than the control males (p=0.03), while the PRO 100 mg/kg bw/day males showed a tendency to longer latency to finding the platform (Fig 6).

The results indicate effects on some of the procymidone exposed groups, as the females exposed to PRO 50 mg/kg bw/day in memory day 2 and the males exposed to PRO 25 and PRO 50 mg/kg bw/day performed better in the new learning. As the males exposed to PRO 100 mg/kg bw/day showed a tendency to a longer latency, there is no effect on new learning that points in the same direction and therefore no dose-response relationship.

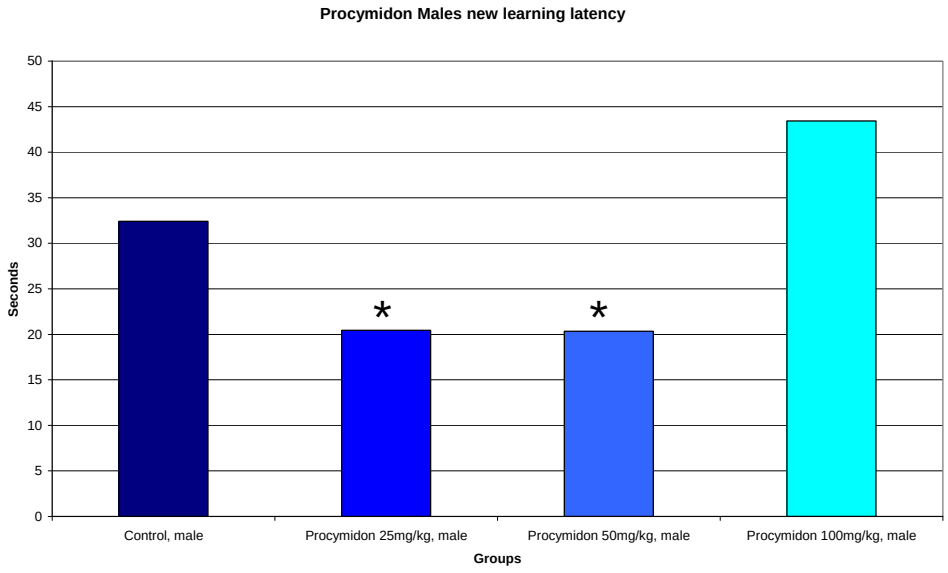


Figure 6. New learning in Morris water maze, males. Only group means are shown. p = 0.05 (indicated by an asterisk) statistically different from control. The PRO 25 and PRO 50 mg/kg bw/day males showed shorter latency than the control males, while the PRO 100 mg/kg bw/day males showed a tendency to longer latency to finding the platform.

Sweet Preference

There was a significant gender difference (p=0.04) as females drank more sweetened water than the males (relative to body weight), but no significant effect of exposure on the sweet preference test was found.

Study 4 A mixture of 3 AR antagonists vinclozolin, flutamide and procymidone

In this study, offspring from the following groups were included in the behavioural studies: control, MIX 7.9 and MIX 19.7, and vinclozolin (VIN) 24.5 mg/kg bw/day. The 24.5 mg/kg bw/day was a higher amount of vinclozolin than the amount of vinclozolin in MIX 7.9 and MIX 19.7. The number of animals was 14 males and 14 females in each group, representing 11-13 litters per group. For the play behaviour and the mating behaviour a total of 11-14 pairs were included from each group.

Play behaviour

No significant gender difference or effect of exposure was found and play behaviour was not included in study 5.

Activity adults

No significant gender difference for total activity was found in the control group. The females from VIN 24.5 mg/kg bw/day were more active than the controls (p=0.009), and showed a higher number of break counts (p=0.004). During the first part of the testing period, female activity was significantly higher in both the MIX 19.7 (p=0.02) and the VIN 24.5 mg/kg bw/day (p=0.002) groups compared to female controls (Fig. 7). During the last part of the testing period, the MIX 7.9 males were less active than the male controls (p=0.003), but no significant differences were seen in relation to MIX 19.7 and VIN 24.5 mg/kg bw/day.

The results from the activity test indicated that the females exposed to MIX 19.7 and VIN 24.5 mg/kg bw/day, but not to MIX 7.9 showed hyperactivity compared to control, but this was not significant in the last testing period. The MIX 7.9 males were less active, but this was only significant in the last period and no dose response was seen.

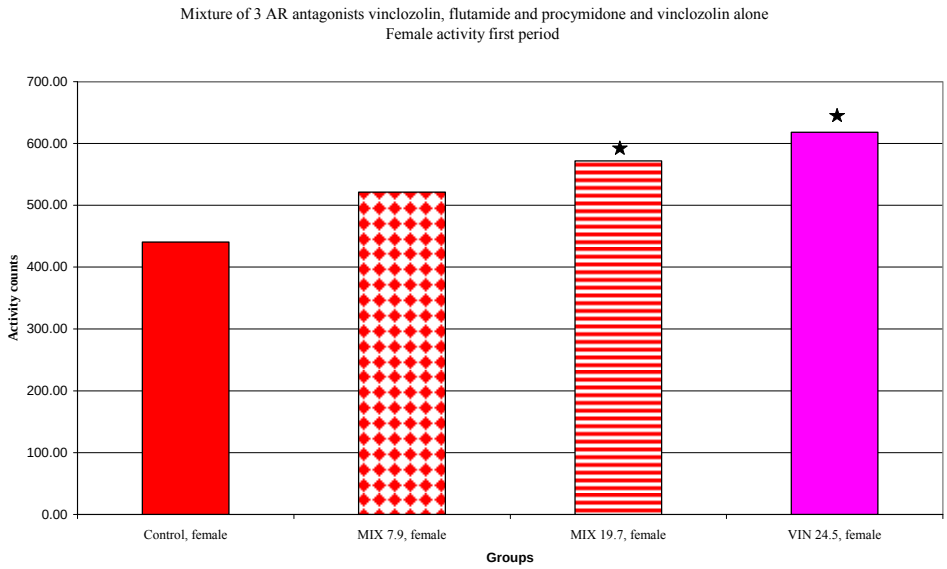


Figure 7. Activity counts in female adults. Only group means are shown. p = 0.05 (indicated by an asterisk) statistically different from control.

Morris water maze

No overall gender difference was seen in the control group during learning and memory testing. During the learning period (day 1 to 5 in total), the males from MIX 19.7 showed increased latency to finding the platform (p=0.045). Both MIX 7.9 and MIX 19.7 males showed increased swim length (p=0.049 and p=0.02) compared to controls in the learning period, while the VIN 24.5 males swam significantly shorter (p=0.02%) than control males (Fig. 8).The results indicate that the learning is impaired in the mixture-exposed males. On the other hand the vinclozolin exposed males (VIN 24.5 mg/kg bw/day) swam statistically significantly shorter than the controls.

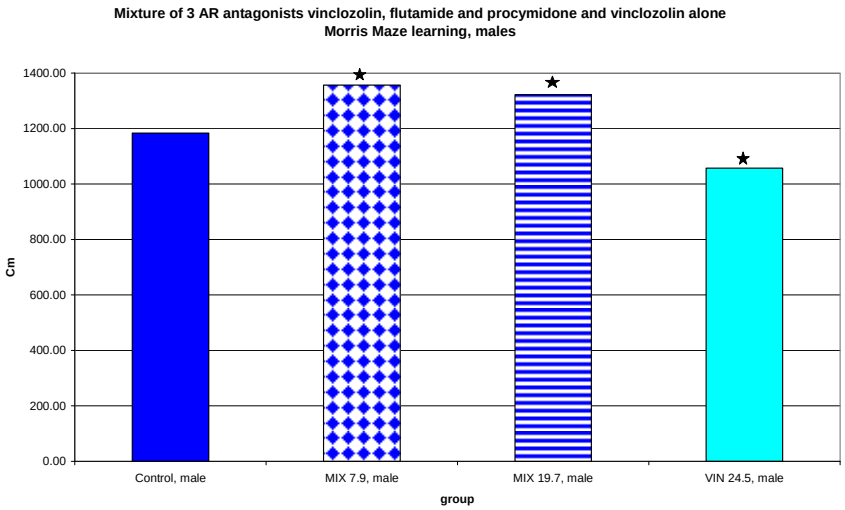


Figure 8. Morris water maze learning (day 1-5 in total) (measured as swim length), males. Only group means are shown. p= 0.05 (indicated by an asterisk) statistically different from control.

Sweet Preference

There was a significant gender difference ($p<0.001$) as females drank more sweetened water than the males (relative to body weight), but no significant effect of exposure was found on this endpoint.

Mating behaviour

A statistic significant effect of exposure was found for the parameter "mounting", as VIN 24.5 mg/kg bw/day males mounted significantly more than control males ($p= 0.04$) (Fig. 9). More of the males from the MIX 7.9 group tended to ejaculate ($p=0.05$) compared to controls and a significantly higher number of the MIX 7.9 males ejaculated ($p=0.014$) compared to VIN 24.5. There was no dose response relationship, as no consistent effect on ejaculation in MIX 19.7 males was observed. No significant difference was observed between the exposed and control groups for the parameter "intromission" in the male rats or the lordosis quotient in the female rats.

The results showed an effect on mating behaviour in the males exposed to vinclozolin alone (VIN 24.5 mg/kg bw/day).

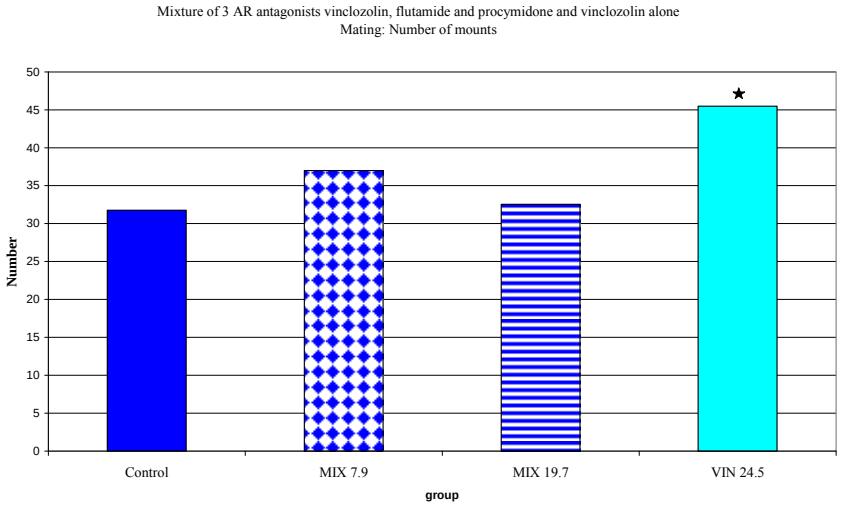


Figure 9. Mating behaviour, number of mounts during a 20 min. period. Only group mean values are shown. $p=0.05$ (indicated by an asterisk) statistically different from control.

Study 5 A mixture of 4 dissimilarly anti-androgens: vinclozolin, finasteride, DEHP and prochloraz

In this study, offspring from the following groups were included in the behavioural studies: control, mixture groups: MIX 13.01; MIX 65.05 and MIX 130.1 (only females because the males in MIX 130.1 were necropsied because of severe malformations). The number of animals was 13-16 males and 14-16 females in each group representing 9-16 litters per group. For the mating behaviour, a total of 13-16 pairs were included from each dose group.

Activity adults

No significant gender difference or effect of exposure was found.

Morris water maze

A significant gender difference was found for most learning endpoints, as the females showed longer latency to finding the platform and lower swimming speeds on most of the days than the males. In the memory period, this gender difference was not observed. In the females, we found a significantly reduced swim speed in MIX 65.05 ($p=0.02$), but this was not seen in MIX 130.1. When only looking at the males, there were no statistical significant effects of exposure on either learning or memory.

The results showed no consistent effect of exposure and there are no findings indicating an effect on learning.

Sweet Preference

There was a significant gender difference ($p<0.0001$) in controls as females drank more sweetened water than the males (relative to body weight). An overall significant effect of exposure ($p= 0.02$) was observed, and the females from MIX 130.1 showed a significant increased ($p=0.04$) intake of sweetened water relative to the controls females (Fig. 10).

The results showed an increased sweet preference in MIX 130.1 females.

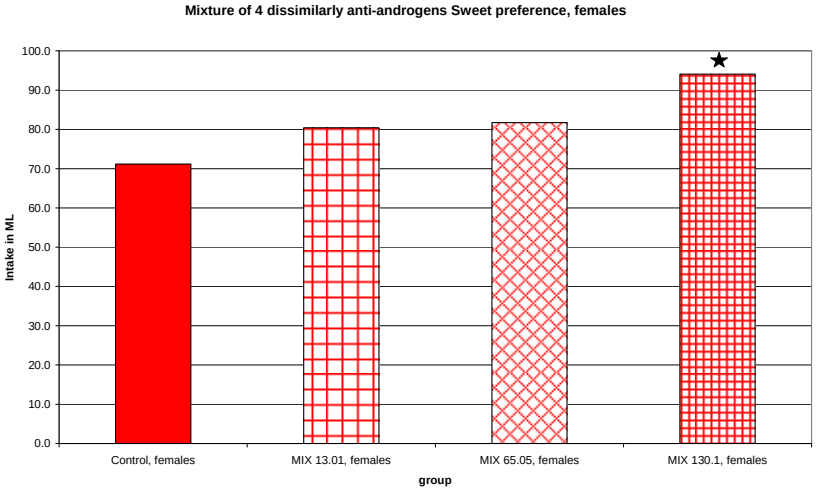


Figure 10. Sweet preference, intake of sweetened water in mL, females. p=0.05 (indicated by an asterisk) statistically different from control.

Mating behaviour

No significant differences in mating behaviour were revealed between the two groups exposed to the mixtures (MIX 13.01 and MIX 65.05) compared to control. Even through we had a high number

of pairs in each group (13-16 pairs), no significant differences for the parameters: “mount”, “intromission” (frequency and latency), “ejaculation” or “lordosis quotient” between control and exposed rats were observed.

Summary of results

Table 2 shows an overview of the results in this thesis and an indication which endpoint was the most sensitive in relation to chemical or mixture of chemicals.

Chemicals	**Development of reproductive external organs (LOAEL)**	**Behavioural (LOAEL)**	**Most sensitive endpoint**
Finasteride (FIN)	At FIN **0.01** mg/kg bw/day effect on NR, while effects on AGD and malformations were seen at 0.1 and 1 mg/kg bw/day, respectively	**FIN 10** mg/kg bw/day decreased activity in females	Nipple retention
Prochloraz (PZ)	**PZ 50** mg/kg bw/day for NR and >100 mg/kg bw/day for AGD	No behavioural effects in relation to PZ 10, 25 and 50 mg/kg bw/day	Nipple retention
Procymidone (PRO)	**PRO 14.1** mg/kg bw/day for NR and **25** mg/kg bw/day for AGD	**PRO 100** mg/kg bw/day females increased play behaviour	Both nipple retention and AGD were more sensitive endpoints than behavioural effects
DEHP	**10** mg/kg bw/day for both NR, AGD	No behavioural effects in relation to DEHP 100 mg/kg bw/day	Both nipple retention and AGD were more sensitive endpoints than behavioural effects
Flutamide	**0.5** mg/kg bw/day for NR	Flutamide exposed rats were not included in behavioural tests	Nipple retention; behavioural effects were not studied
Vinclozolin (VIN)	**5** mg/kg bw/day for NR and 10 mg/kg bw/day for AGD	**VIN 24.5 mg/kg bw/day** -females were more active -males swam shorter in the learning period - males mounted significantly more	Nipple retention
MIX 3 AR antagonists (Vinclozolin, Flutamide, Procymidone)	**MIX 7.9** mg/kg bw/day for NR and weight of epididymides **MIX 19.7** mg/kg bw/day also for ventral prostate and bulbouretral glands **MIX 39.3** mg/kg bw/day for effect on AGD	**MIX 7.9** and **MIX 19.7** learning impaired in males in Morris water maze	Behaviour was just as sensitive as the effects on the reproductive organs
MIX 4 dissimilar acting anti-androgens (Finasteride, Prochloraz, Vinclozolin, DEHP)	**MIX 13.01** mg/kg bw/day for effect on NR and AGD, while MIX 65.05 mg/kg bw/day showed malformations	**MIX 130.1** mg/kg bw/day females showed increased intake of sweet preference	Both AGD, nipple retention and malformations were more sensitive endpoints than behavioural effects

Table 2 shows an overview of the LOAELs (Lowest adverse effect level) in this thesis and an indication which endpoint was the most sensitive in relation to a specific chemical or a mixture of chemicals.

Discussion

The physiological anti-androgenic effects of the anti-androgen exposure were very clear in the male offspring – most of the exposed male animals had reduced anogenital distances, retained nipples and some of the dose groups showed external malformations of the reproductive organs. The anti-androgen exposure also affected the sexually dimorphic behaviour in some of the behavioural tests, but the effects were more complex. In some cases the results indicated a masculinised behaviour whereas in other cases incomplete masculinisation was indicated.

This complexity of exposure effects could be due to the fact that the behavioural tests in these studies frequently lacked the otherwise expected gender differences. In these cases where the behavioural tests did not show significant gender differences in the control groups the test would likely not be especially sensitive to show effects on sexual dimorphic behaviour.

A reason for the different sensitivity of morphological versus behavioural measures (table 2) could be that although the group sizes (i.e. number of litters) are similar, the number of pups examined pr. litter for behavioural effects is lower than those examined for morphological effects before weaning. To increase the sensitivity of behavioural measures it could be an advantage to include more than 1 rat pr. litter.

Below the results for effects of both single chemicals and mixture of chemicals are presented.

Finasteride

Effects on both motor activity, and learning and memory were found after exposure to finasteride. The decreased activity observed in females in two of the finasteride exposed groups (0.1 or 10 mg/kg bw/day) indicates incomplete feminisation in relation to the activity behaviour in females. In the learning and memory test (Morris water maze), the exposed males showed both improved initial learning (i.e. masculinisation), but also impairment of new learning ability (i.e. incomplete masculinisation). In both cases, however, there was no clear dose-response relationship and therefore, the findings are inconclusive.

No references were found in literature concerning pre- and postnatal exposure to finasteride and effects on motor activity, or learning and memory.

Behaviour was not the most sensitive endpoint regarding pre- and postnatal exposure to finasteride, as the male rats showed nipple retention at a 1000 times smaller dose and AGD was reduced at a 100 times smaller dose (Table 2).

Prochloraz

No significant effects of exposure to prochloraz (10, 25 and 50 mg/kg bw/day) were found on any of the behavioural endpoints tested. Furthermore, no significant effects of exposure were seen in the mixture study with four dissimilarly acting anti-androgens, where prochloraz was present at doses of 5 and 25 mg/kg bw/day.

Only Vinggaard et al., from our laboratory, have reported effects on behaviour after perinatal exposure to prochloraz (Vinggaard et al. 2005) Pregnant rats were exposed to 30 mg/kg bw/day of prochloraz or a mixture containing prochloraz (20 mg/kg bw/day) and 4 other pesticides (each in a dose of 1.25 mg/kg bw/day). They found a statistically significant gender difference in the motor activity test, and reported that the prochloraz-exposed adult males showed a statistically significantly increased level of motor activity. The sweet preference test showed a clear gender difference, with females exhibiting higher preference for sweetened water than males. The males exposed to prochloraz (30 mg/kg bw/day) generally showed a higher preference than control males during the 3 days of the test, but the differences were only statistically significant on the second day. The findings in Vinggaard et al. could not be repeated in the studies included in this thesis. The reason for not being able to repeat the findings remains unanswered as we in the behavioural testing included a higher dose of prochloraz (50 mg/kg bw/day) than was included in Vinggaard et al. (30 mg/kg bw/day) (Vinggaard et al. 2005).

Behaviour was not the most sensitive endpoint with regard to pre- and postnatal exposure to prochloraz, as the male rats showed nipple retention at 50 mg/kg bw/day where we did not observe any behavioural effects of exposure (Table 2).

Procymidone

Increased play behaviour was found in females exposed to PRO 100 mg/kg bw/day compared to the control females. This indicates a masculinising effect of procymidone in the females. In the Morris water maze, PRO 50 mg/kg bw/day females (memory day 2) performed better (i.e. masculine direction), but no dose response relationship was found, as no consistent effect on memory of PRO 100 mg/kg bw/day females was observed. In the Morris water maze, PRO 25 mg/kg bw/day and PRO 50 mg/kg bw/day males (new learning) performed better (i.e. masculine direction). However, in contrast to this, PRO 100 mg/kg bw/day males showed longer latency (i.e. feminised direction) so no consistent effects or direct dose-response relationship were found.

Numerous studies have shown that rough-and-tumble play is a sexually dimorphic behaviour that is affected by the levels of pre- and postnatal sex hormones. There is general agreement that play

behaviour is one of the few sexually dimorphic behaviours where the behaviour of the males is organized by the testosterone itself and not by oestradiol aromatised from testosterone in the brain (Meaney and Stewart 1981; Meaney et al. 1983; Gray and Ostby 1998). Since PRO exerts its anti-androgenic actions by binding to the androgen receptors, we expected the prenatal PRO exposure to result in demasculinisation of male and female play behaviour. We found an indication of the opposite effect in females, but the results from other studies where play behaviour has been tested after perinatal anti-androgenic exposure have been ambiguous as well.

No references were found in literature on the topic of pre- and postnatal exposure to procymidone and effects on play behaviour and learning and memory.

Behaviour was not the most sensitive endpoint when evaluating pre- and postnatal exposure to procymidone, as the male rats showed nipple retention at an almost 10 times lower dose level (14.1) and reduced AGD at a 4 times lower dose level (PRO 25) than where the behaviour was affected (increased play behaviour in females) (Table 2).

Vinclozolin

Increased activity was observed during the initial part of the period in females exposed to VIN 24.5 compared to control females.

In the Morris water maze, the VIN 24.5 males swam significantly shorter than control males and in mating behaviour, a significantly increased number of mounts were seen in VIN 24.5 exposed males.

Despite the fact that it has been known for a long time that the activity level is a non-reproductive behaviour, organised by gonadal hormones during development (Dawson et al. 1975; Beatty 1979), only few studies have tested the effects on activity after developmental exposure to anti-androgenic compounds. Flynn et al. tested the effects of perinatal exposure to vinclozolin (60 mg/kg bw/day dosed from GD7-PND77) on running wheel and open field motor activity levels (Flynn et al. 2001). In the open field, they did not find any significant effects of vinclozolin exposure, neither in males nor in females. However, they did not find the expected gender differences. In the running wheel activity test, Flynn et al. did show a gender difference, but still did not observe any significant effects on the activity levels of the males (Flynn et al. 2001). They did, however, find a decreased running wheel activity in the females, which they explained by a possible masculinising effect of vinclozolin when dosed in adulthood (activational rather that organisational effect). This result could, however, also fit our theory about the masculinising effect on the brain by perinatal anti-androgen exposure.

The differences between our results and the results from Flynn et al. may be due to differences in the methods used (Flynn et al. 2001). Even though both studies have tried to assess the activity levels of animals exposed perinatally to an anti-androgenic compound, no clear picture has been found. This is probably due to the many differences in the experimental setups. First of all, the two studies used different dosing periods, pre- and postnatal vinclozolin exposure versus exposure until adulthood. Another important difference was the method used to assess activity level (open field and running wheel vs. activity box). This is obviously quite an important parameter, since the results from the Flynn et al. study showed different results depending on the activity measurement used, even though all the other parameters were the same (Flynn et al. 2001). In conclusion, it is at present difficult to predict what happens to the activity level of male and female animals perinatally exposed to anti-androgenic compounds, but nothing points in the direction of a demasculinising effect on the males.

In literature it has been reported that male rats which have morphological abnormalities of the external genitalia due to maternal exposure of vinclozolin show disturbances of mating behaviour such as reduces intromissions, ejaculation, and consequently reduced fertility despite no changes in mounting behaviour (Gray et al. 1994). The dosing period and dose level are not comparable with the one in our study, and all males in the study by Gray et al. showed malformations of the external sex organs (Gray et al. 1994).

Behaviour was not the most sensitive endpoint regarding pre- and postnatal exposure to vinclozolin, as the male rats showed nipple retention at 5 mg/kg bw/day and reduced AGD from 10 mg/kg bw/day. The behavioural studies did not include lower doses of vinclozolin than the dose VIN 24.5 mg/kg bw/day, where behavioural effects were observed (Table 2). The females' behaviour pointed in the direction of a feminised effect (more active) while the males' behaviour pointed in the direction of a masculinising effect (increased learning, increased mounting).

Mixture findings

Mixture of 3 AR antagonists

Increased activity was observed, during the initial part of the period in females exposed to MIX 19.7 compared to control females. The males exposed to MIX 7.9 were less active (i.e. masculinised), but this was only significant in the last period. Furthermore, no dose response relationship was observed, as no effects were observed on males exposed to MIX 19.7.

Learning was impaired (i.e. feminised effect) in males dosed with both MIX 7.9 and MIX 19.7 when tested in the Morris water maze. In these groups, no significant effects were seen on the developmental endpoint AGD, but effects were seen for nipple retention at both MIX 7.9 and MIX 19.7((Hass et al. 2007), paper II). The males exposed to MIX 7.9 showed significantly decreased weights of epididymis, while the males exposed to MIX 19.7 showed decreased weights of epididymis, ventral prostate and bulbouretral glands as reported in Metzdorff et al.((Metzdorff et al. 2007), paper III). The malformations seen in MIX 7.9 and MIX 19.7 groups were few and mild both at PND 16((Metzdorff et al. 2007), Paper III) and PND 47((Christiansen et al. 2008), paper IV).

A tendency to an increasing number of ejaculating males from MIX 7.9 was observed, but this was not seen in MIX 19.7. Apart from that, no effects of the exposure were seen on mating behaviour in the mixture-exposed rats, while a significantly increased number of mounts were seen in VIN 24.5. The dose of vinclozolin used in this group is 24.5 mg/kg bw/day, while it is present in doses of 4.9 mg/kg bw/day and 12.2 mg/kg bw/day in MIX 7.9 and MIX 19.7, respectively. The doses could easily be converted to flutamide equivalents (as the mixture is based on the potency of vinclozolin: flutamide; procymidone at the ratio; 31:1:18). The VIN 24.5 consists of 0.77 flutamide equivalents, while in the MIX 7.9 and MIX 19.7 VIN is present in doses of 1.16 and 0.47 flutamide equivalents, respectively. Thus, VIN 24.5 is in between the two mixture doses according to potency. In this study, neither the metabolism of the 3 anti-androgens nor their ability to penetrate the blood-brain barrier was investigated, which could have provided possible explanations for not finding a significant effect of the mixture exposure in relation to mating behaviour. Another possible explanation of the lack of effects could be that testosterone was elevated in the male rats. In some studies, treatment with another AR antagonist, flutamide has resulted in increased levels of LH and testosterone in the blood. This effect has been seen several times after flutamide treatment of adult rats (Södersten et al. 1975; Viguier-Martinez et al. 1983), but also prepubertal (Rulli et al. 1995), and neonatal (Pakarinen et al. 1994) flutamide treatment has been shown to have this effect. The elevated levels of LH and testosterone found in these experiments can be explained by the fact that AR antagonists blocks the androgen receptors in the brain, thereby reducing the negative feedback that the endogenous testosterone exerts on the hypothalamus and pituitary, which in turn will result in increased LH and testosterone production.

If receptor mediated anti-androgenic exposure during the prenatal period of brain development has the overall effect of higher circulating levels of testosterone, this could lead to an increased amount

of substrate for aromatisation, and thereby increased availability of oestradiol in the brain. If this is what happened to the anti-androgen exposed animals in our studies, it can explain why we, in some of the behavioural tests, did not see demasculinisation of the male offspring - and in some of the tests even saw a masculinising effect of anti-androgen (receptor mediated) exposure (e.g. more mating activity).

Some of the behavioural tests showed that behaviour could be just as sensitive an endpoint as the development of reproductive organs after exposure to combinations of 3 AR antagonists (vinclozolin, flutamide, and procymidone). The learning in Morris water maze was impaired at the same dose levels where effects on nipple retention, and weight of epidydimides, ventral prostate and bulbouretral glands were seen.

Mixture of 4 dissimilarly anti-androgens

The only effect on behaviour observed in this study was that the MIX 130.1 females showed an increased intake of sweet water compared to control females. This could be evaluated as increased feminisation in this group. Behaviour was not the most sensitive endpoint regarding pre- and postnatal exposure to a mixture of 4 dissimilarly acting anti-androgens, as the male rats showed nipple retention and reduced AGD at a 10 times lower dose level (MIX 13.01 = Mixture of NOAELs) than where the effects on female behaviour were seen (MIX 130.1, effects on sweet preference in females).

Short conclusion

In conclusion, it is at present difficult to predict what happens to the behaviour of male and female rats pre- and postnatally exposed to anti-androgenic compounds. In some cases, the effect observed points in the direction of a masculinising effect on the males and a feminised effect on the females, while the physiological anti-androgenic effects are demasculinised male organs. The behavioural tests used in this thesis commonly lacked the expected gender differences in the control group and this may have contributed to the lack of clear exposure effects. Consequently, the methods needs to be further developed and modified to obtain the expected gender differences in the future.

A part of this thesis was to develop and use a new test for mating behaviour in rats. This method development is still ongoing and the applicability can therefore not be evaluated at present. There is a need for further studies and also for validation of chemicals known to affect mating behaviour. The work will continue in the new EU project CONTAMED with mixtures of anti-androgens and estrogens.

In most of the studies in this thesis, the development of reproductive organs was a more sensitive endpoint than behaviour. Nevertheless, animal behaviour (learning in Morris water maze) seemed to be just as sensitive as the development of reproductive organs when testing the mixtures of 3 AR antagonists (in study 4). Thus, in future testing of chemical mixtures it will be very relevant to look also at behavioural effects as these results could contribute to a broader picture of the toxicity of mixtures of EDCs than studies that only takes the development of reproductive organs into account.

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Paper I

Christiansen S, Boberg J, Axelstad M, Dalgaard M, Vinggaard AM, Metzdorff SB & Hass U. Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats *In preparation*

Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats

Sofie Christiansen*, Julie Boberg, Marta Axelstad, Majken Dalgaard, Anne Marie Vinggaard, Stine Broeng Metzdorff and Ulla Hass

National Food Institute, Technical University of Denmark,
Dept. of Toxicology and Risk Assessment,
Mørkhøj Bygade 19
DK-2860 Søborg
Denmark
Tel.: +45 72 34 70 25
*) To whom correspondence should be addressed. Fax: + 45 72 34 76 99,
E-Mail: sochr@food.dtu.dk

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Running title

Anti-androgenic effects in rats after DEHP exposure

Title

Low-Dose Perinatal Exposure to Di(2-ethylhexyl) Phthalate induces Anti-androgenic Effects in Male Rats

Abstract

Developmental exposure to di(2-ethylhexyl) phthalate (DEHP) demasculinizes male rat offspring by reducing anogenital distance (AGD), causing nipple retention (NR), and malformations and weight reductions of some reproductive organs.

We investigated the effects of perinatal DEHP exposure in part A and B from a study, in which time-mated Wistar rats were gavaged from gestation day 7 to postnatal day 16 with 0, 10, 30, 100, 300, 600 and 900 mg/kg bw/day and 0, 3, 10, 30 and 100 mg/kg/day, respectively. The dose levels were selected with the aim of covering the whole dose-response curve for the demasculinizing effects of DEHP as well as investigating low dose effects.

Our results demonstrate that DEHP at a relatively low dose of 10 mg/kg causes adverse anti-androgenic effects on male rat development. At this dose level, male anogenital distance was decreased, the incidence of nipple retention was increased, weight of *levator ani/bulbocavernosus muscle* was reduced and mild external genitalia dysgenesis was observed. Higher doses of DEHP, i.e. from 100 mg/kg, additionally induced histopathological effects on the testes, reduced testicular and prostate weight, and reduced expression of androgen-regulated genes (PBP C3 and ODC mRNA) in the prostate.

The results provide new evidence of low-dose effects of DEHP, supporting that the cautious NOAEL of 5 mg/kg for DEHP that has been decided in EU is appropriate. Our results also indicate a reason for concern about the current exposure level of the human population.

Key words Diethylhexyl phthalate, DEHP, low-dose, phthalates, endocrine disrupters, developmental toxicity, rats

Introduction

Di(2-ethylhexyl) phthalate (DEHP) is a high production volume chemical. It is used as a plasticizer in a wide range of consumer products, including vinyl floors, food wrapping materials, cosmetics, medical products and toys, making it one of the phthalates most abundantly found in the environment[1,2,3] . It is well known that humans, and in particular children, are exposed to phthalates in considerable doses and the mean adult phthalate exposure to DEHP, DBP, DINP and DIDP collectively is presently estimated to be approximately 0.16 mg/kg bw/day)[3]. For DEHP alone, adult exposure, via the environment, is calculated to be maximally 0.02 mg/kg/day, while the maximal exposure level for a small child is 0.10 mg/kg/day [3]. The exposure in the children is calculated to be 5 times higher primarily because of the expected exposure from PVC toys.

Recently published studies reveal the presence of various phthalate monoesters in breast milk, including mono(2-ethylhexyl) phthalate (MEHP) the primary metabolite of DEHP, and presence of some of these phthalate monoesters seems to be correlated with increased levels of sex hormone-binding globulin (SHBG), luteinizing hormone (LH) and an increased ratio of LH/Free testosterone in boys at three month of age [4]. Furthermore, Swan et al have reported associations between phthalate exposure and shortened anogenital index (AGD/weight) in infant boys whose mothers had elevated urine levels of phthalate metabolites during pregnancy [5]. These studies clearly show that humans are exposed to phthalates during sensitive periods of perinatal development and indicate that exposure to phthalates may cause developmental effects in human infants.

Numerous rodent studies have shown clear anti-androgenic effects caused by developmental exposure to DEHP at medium and high levels, i.e. exposure above 100 mg/kg [6-9], but only few rat studies have investigated DEHP at lower doses [10-12].

Developing and prepubertal rats have been found to be more sensitive to DEHP than adult rats, as young animals have shown adverse effects after exposure to much lower doses than adults [12-14]. Moreover, gestation and lactation are even more sensitive periods, and exposure of rats during these periods can cause irreversible effects at dose levels resulting in minimal effects in adult animals [12,15].

Normal male masculinization and development is dependent on the presence of the two androgens, testosterone and 5α-dihydrotestosterone (DHT). The specific development of the male external genitalia as well as the ventral prostate is DHT-dependent [16]. DHT is also responsible for apoptosis of nipple anlagen in male rats, causing lack of nipple development. Therefore, male rats do not normally exhibit nipples or areolas as female offspring do. Furthermore, DHT is responsible for the development of external male genitalia and for growth of the perineum to produce a normal male anogenital distance (AGD), which is defined as the distance between the anus and the genital bud [16-19]. At the time of birth, AGD in male control rats is approximately twice as long as it is in females. AGD and nipple retention (NR) are two very sensitive and non-invasive endpoints, when investigating anti-androgenic compounds during the critical periods of prenatal development [20-23]. In addition to the effects on AGD and nipple retention, the anti-androgenic action of DEHP also affects the development of reproductive organs. Testicular morphology is affected [12,15] and the weights of androgen-dependent organs such as the ventral prostate and seminal vesicle are reduced [11,24].

The most likely mechanism by which DEHP exerts its demasculinizing effects seems to be the lowering of testosterone production towards female levels in male rat foetuses during critical stages of sex differentiation [6,9,25,26]. Whether DEHP and MEHP also act as anti-androgens by antagonising the AR receptor is still debated. Some studies have shown that

neither DEHP nor MEHP are androgen receptor antagonists, as they do not compete with androgens for binding to the AR at concentrations up to 10 μM [5,7,27] while another study has shown that metabolites of MEHP do act as AR antagonists *in vitro* [28]. The aim of our work was to study the developmental toxicity of DEHP in rats, and especially to focus on low dose effects. Two studies were performed, in which dams were dosed with different doses of DEHP during the gestation and lactation period. The purpose of part A was to cover the whole dose-response curve, including a dose level where no effects were expected. The doses were: 10, 30, 100, 300, 600, 900 mg/kg. The results from this part, somewhat unexpectedly, showed anti-androgenic effects at the lowest dose level (10 mg/kg/day). Consequently, it was decided to repeat the three lowest doses in a second part (B) and to include a lower dose of 3 mg/kg.

In both parts of the study measurements of AGD and NR in male rat offspring were performed, along with investigations of other endpoints found to be sensitive to anti-androgenic chemicals in male offspring. These were weights and histopathology of reproductive organs, malformations of male external genitals and the expression of androgen-regulated genes in the prostate. To further investigate the testicular effects of DEHP, immunohistochemical staining for Sertoli cell specific markers was performed, and serum levels of inhibin B was measured as a marker of Sertoli cell function.

Results from both DEHP studies and from a combination of the two studies are reported here.

Materials and Methods

Chemicals. DEHP (di(2-ethylhexyl) phthalate), CAS No. 117-81-7, purity 99% was obtained from VWR & Bie & Berntsen (Herlev, Denmark). DEHP was dissolved in corn oil (VWR & Bie & Berntsen), which was used as the vehicle.

Animals and exposure. In part A, 64 time-mated nulliparous, young adult Wistar rats (HanTac: WH, Taconic Europe, Denmark, body weight approximately 200 g) were supplied at day 3 of pregnancy. The day following mating was designated gestational day (GD) 1, and postnatal day (PND) 1 was the day of birth. The dams were, at the day after arrival i.e. GD 4, randomly distributed according to body weight (bw) into a control group of 16 dams and 6 DEHP groups of 8 dams. The dams were housed in pairs until GD 21 and single-housed thereafter under standard conditions; semitransparent plastic cages (15x27x43cm) with Aspen bedding (Tapvei), situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lux, temperature 21 ± 2°C, humidity 50% ± 5%, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) was given to all dams. Acidified tap water (to prevent microbial growth) was provided *ad libitum*. An acclimatization period of 4 days was allowed before the exposure-period started. The animals were weighed every day to calculate the dosing volume of 2 ml/kg bw and they were gavaged with vehicle (corn oil) or 10, 30, 100, 300, 600, or 900 mg DEHP/kg bw/day (mg/kg) from GD 7 to the day before expected birth (GD 21), and from the day after birth (PND 1) until PND 16. This day was chosen as the last day of dosing, because the pups graduately stop suckling and starts eating at this age. Animals were inspected for general toxicity twice daily. Part A was divided into 4 blocks (with one week in between), and each dose group was represented equally in all 4 blocks.

In part B, 56 time-mated nulliparous, young adult Wistar rats (HanTac: WH, Taconic Europe, Denmark, body weight approximately 200 g) were supplied at day 3 of pregnancy. The dams were housed similarly as in study 1, and also similarly distributed into 4 blocks. At GD 4, the dams were randomized according to weight into 5 groups of either 16 dams (control group and the group dosed with 3 mg/kg) or 8 dams per group (10, 30, or 100 mg DEHP/kg) and dosed during the same exposure periods and conditions as in part A. Number of rats in the different groups and in the two studies is mentioned in table 1.

Postnatal development. After birth (PND 1), all live pups in the litter were weighted, sexed and anogenital distance (AGD) was measured using a stereomicroscope. At PND 12±1 all pups were examined for the presence of nipples/areolas (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. The nipples were counted and no distinction was made between the retention of an areola or a nipple. AGD and NR was recorded blindly to exposure group by the same technician in both studies.

Autopsy of offspring on PND 16. The male offspring underwent a thorough autopsy at PND 16. Male external genitals were investigated, and the organs listed below were excised, weighed, and used for either histopathological investigation or gene expression analysis. Furthermore, trunk blood was taken from all male pups and analyzed for inhibin B in serum as described in [6]. Blood samples were pooled within litters.

Investigation of male external genitals. The external genitalia were inspected blinded to the observer at PND 16 in all males from all litters. The changes were scored on a scale from 0 to 3 in order to investigate whether male external genitals were demasculinized. The following criteria were used:

Score 0, no effect: normal genital tubercle, the urethral opening is found at the tip of the genital tubercle and the preputial skin is intact. In the perineal area, thick fur extends caudally from the base of the genital tubercle and half the distance to the anus. A furless area circumscribes the anus.

Score 1, mild dysgenesis of the external genitalia: a small cavity on the caudal surface of the genital tubercle or a minor cleft in the preputial opening is observed, estimated 0.5-1.4 on an arbitrary scale. The furless area around anus expands towards the base of the genital tubercle, but thick fur is still present at the base of the genital tubercle.

Score 2, moderate dysgenesis of the external genitalia: the preputial cleft is larger, estimated 1.5-2.4 on an arbitrary scale. The urethral opening is situated half way down the inferior side of the genital tubercle (hypospadia). Partly furless e.g. thin fur is noted in the perineal area ranging from the base of the genital tubercle and caudally to the furless area circumscribing the anus.

Score 3, severe dysgenesis of the external genitalia: The preputial cleft is large, estimated 2.5-3.5 on an arbitrary scale. The urethral opening is situated further than half way down the inferior side of the genital tubercle to the base of the genital tubercle. At the base of the genital tubercle a groove extending laterally is observed (similar to control females at PND 16). The rat is totally furless in the whole perineal area.

Organ weight, histopathology, and estimation of seminiferous tubule diameter. Body weights of all male pups were recorded. In one male per litter the following organs were excised and weighed: liver, kidneys, adrenals, testes, epididymides, seminal vesicles, ventral prostate, bulbourethal glands, and the levator ani/bulbocavernosus muscles (LABC). In the analysis of body and testis weight generally one to four males per litter were used.

From 1 or 2 males per litter, the right or left testes were alternately fixed in Bouin's fixative, paraffin embedded, and stained with haematoxylin and eosin. The contra lateral testis was fixed in formalin and used for immunohistochemistry. The following organs were fixed in formalin: epididymides, seminal vesicles and ventral prostate. All fixed organs were embedded in paraffin and examined by light microscopy after staining with haematoxylin and eosin. Generally, testis histopathology was investigated in one to two males per litter. Diameters of the seminiferous tubules were investigated semiquantitatively in one cross section per testis in one to two males litter (in part A, n=16 in the control group, n=8 in the dosed groups, in study 2, n=8 in all groups). The diameter was estimated by systematic random sampling of 80-100 seminiferous tubules per testis cross section using a computer assisted microscope (Olympus BH2, Denmark) described in details by [29].

Immunhistochemistry Vimentin, Androgen receptor (AR), and Antimüllerian hormone (AMH). Immunohistochemistry was performed on one section per testis. Following microwave pre-treatment for either 2x5 min (vimentin and AR) or 1x5 min (AMH) in citrate buffer pH 6 at 100^{o}C, sections were blocked for endogenous peroxidase activity in 3% H_2O_2 in PBS, and blocked in 1% bovine serum albumin in PBS. Sections were then incubated over night at 4˚C with the following antibodies: vimentin 1:2000 (clone V9, DAKO), AMH 1:8000 (also called Müllerian inhibiting substance, MIS, DAKO), or AR 1:200 (N20SC816P, Santa Cruz).

Sections were then incubated for 30 min with secondary antibody anti-mouse EnVision+ (DAKO) for Vimentin, and rabbit anti-goat antibody (DAKO) enhanced with anti-rabbit EnVision+ for AMH staining, and anti-rabbit EnVision+ for AR. Sections were stained in diaminobenzidine (DAB+ Substrate Chromogen System, DAKO, Glostrup, Denmark) and counterstained in Meyer's haematoxylin.

Gene expression levels determined by real-time RT-PCR. Ventral prostate from one male per litter were weighed and stored in RNAlater (Qiagen) for gene expression analyses. Total RNA was isolated using RNeasy-mini kit and RNase-Free DNase set (Qiagen), and cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen). Expression levels of the two androgen-regulated genes: prostate binding protein subunit C3 (PBPC3) and ornithine decarboxylase (ODC) were quantified on the 7900HT Fast Real-Time PCR System (Applied Biosystems) by standard TaqMan technology using TaqMan Fast Universal PCR Master Mix. Expression levels of each target gene were normalized to the expression level of the housekeeping gene 18S rRNA. All genes were quantified from standard curves. Primers and probes for PBP C3, ODC and 18S rRNA have previous been published in [30].

Statistical analysis. Data were examined for normal distribution and homogeneity of variance, and if relevant, data were ln-transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall-Wallis test was used, followed by Wilcoxon's test for pair wise comparison. Statistical analysis of the effect of DEHP dosing on macroscopic lesions, histopathology, vimentin staining and incidence of litters and pups with nipple/areola retention > 2, were done using Fisher's Exact Test.

For statistical evaluation of gene expression data, AGD, NR, body weights and organ weights, one-way ANOVA was employed for all groups. Dunnett's test was performed to determine differences between treated and control group means. When more than one pup from each litter was examined, statistical analyses was adjusted using litter as an independent, random and nested factor in ANOVA. When we pooled data from both parts, 'study' was included in the statistical analysis as a dichotomous factor. Pooled data are only

reported if no significant effect of ‘study’ and no interaction between ‘study’ and ‘dose’ were observed. Data analysis of AGD included the cubic root of body weight as a covariate in the analysis, to correct for the relationship between body size and AGD. In the analysis of organ weights PND 16, body weight was used as a covariate. For the analysis of birth weight, AGD and NR data from all offspring were analyzed. For the analysis of terminal body weight and organ weights PND 16, 1-4 males/litter were included, while 1-2 males/litter were included in the analysis of the diameter of the seminiferous tubules. Generalized linear models (GLM) in combination with generalized estimating equations in order to account for the nested litter correlation analyzed the number of nipples. P-value adjustments were carried out by the ROM procedure, a powerful step-up procedure for protecting the global error rate $\alpha = 5\%$ [31]. Asterisks in tables and figures, indicate a statistically significant difference compared to controls *: $p \leq 0.05$; **: $p < 0.01$. Gene expression data were analyzed in Sigma stat version 2.0. All other analyses were done using the SAS procedure PROC GLM, PROC MIXED, PROC GENMOD and PROC MULTTEST (SAS version 8, SAS Institute Inc, Cary, NC, USA).

Results

Pregnancy data, postnatal growth and general toxicity. In both studies, no general toxicity was observed. Pregnancy and litter data from part A are presented in Table 2a. DEHP had no effect on the maternal weight gain from GD 7 to GD 21 or on the maternal weight on PND 1, and the mean pregnancy length was also unaffected. Significant decreases in the male birth weights were seen at 300, 600 and 900 mg/kg and in the females at 900 mg/kg. On PND 12±1 the body weight of the males was significantly higher at 100 mg/kg and significantly decreased at 900 mg/kg as seen in table 2a, whereas no significant effect on the weight of the females was seen. No effects on sex ratio or postimplantation - perinatal loss were detected. Pregnancy and litter data from part B are presented in Table 2b. In this part, no effects on maternal and pup weights, litter size or pregnancy length was observed, but the postimplantation-perinatal loss was significantly elevated at 10 mg/kg as seen in table 2b.

Anogenital distance (AGD). The anogenital distance is normally approximately twice as long in male as in female control offspring. However, in part A, the prenatal exposure to DEHP significantly decreased AGD in male offspring in a dose-related manner (Table 2a). In part B, AGD was significantly different from control values at 100 mg/kg (Table 2b). Analysis of the combined data showed that prenatal exposure to DEHP significantly decreased AGD in male offspring at all dose levels of DEHP above 3 mg/kg (Figure 1).

Nipple retention (NR). The number of nipples in female rats is normally 12, versus zero in males, but in part A, perinatal DEHP exposure induced NR in male offspring at all dose levels. However, the dose-response relationship was unusual, as 10 mg/kg induced a more marked effect than 30 and 100 mg/kg (Table 2a). In part B, there appeared to be a higher number of nipples at 10 and 100 mg/kg (Table 2b), although the difference was not statistically significant.

The combined analysis of data from both studies showed that, at doses above 3 mg/kg, an increased NR was observed (Figure 2). The combined dose response curve remained unusual, as the dose of 10 mg/kg still seemed to induce a more marked effect than 30 and 100 mg/kg.

The incidence of litters and offspring with NR above 2 is shown in Table 3. When the data from the two studies were combined, significant effects were found at 10 mg/kg when litter was the statistical unit, and at all dose levels, from 3 mg/kg to 100 mg/kg, when the individual pup was used as the statistical unit.

Incidence of mild changes of the male external genitals. Mild dysgenesis of the external genitals (score 1) was observed in all groups (Table 4). When the two studies were combined the incidence of mild dysgenesis was significantly increased at 10 and 100 mg/kg, but not at 300 mg/kg.

Muscle and organ weights. LABC was very sensitive to DEHP and in part A, the weight of LABC was statistically significantly decreased from 10 mg/kg and in part B this weight was decreased at 10 and 30 mg/kg (Table 5). When combining the data from both studies, a significant decrease in weight of LABC was found at all doses above 3 mg/kg. Testis weights were reduced in part A at 100, 600, and 900 mg/kg (left testis) and at 600 and 900 mg/kg (right testis) (Table 5). A reduction in the weights of the adrenals was found in part A at 10, 100 and 900 mg/kg. The liver weight was significantly elevated in part A at 300, 600 and 900 mg/kg.

Histopathological effects. In part A, testis histopathology was investigated in one section from 1 to 3 males per litter in all dose groups. No histopathological effects were observed in the groups receiving 10, 30, or 100 mg/kg and the diameters of the seminiferous tubules were comparable to controls. At 300, 600, and 900 mg/kg, the testes were more immature

with a delay in the development of the seminiferous epithelium (figure 3A and 3B). Fever germ cells were present and focal Leydig cell hyperplasia was observed. These effects were most pronounced in the group receiving 900 mg/kg. The diameter of the seminiferous tubules was decreased dose-dependently from 300 mg/kg (Table 5 and figure 3A and 3B). In testes from part B, no dose-related histopathological changes caused by DEHP were observed. No histopathological effects were observed in epididymides, ventral prostate or seminal vesicles in part A and therefore these organs were not investigated in part B.

Immunohistochemistry. Immunohistological staining of vimentin in testis was scored by an investigator blinded to treatment groups. The high dose group (900 mg/kg) had a more intensive staining, which extended more diffusely throughout the cytoplasm of the Sertoli cells in 6 out of 9 male testes compared to 1 out of 9 in controls (Figure 3C and 3D). This effect was only observed in one testis in the group dosed with 600 mg/kg and therefore vimentin staining was neither investigated in the groups receiving lower doses in part A nor in any of the groups in part B. No effect on testis AR or AMH was observed in any of the dose groups.

Gene expression levels. Expression of the androgen-regulated genes PBP C3 and ODC in PND 16 males was investigated in ventral prostate (Figure 4). In part A, PBP C3 mRNA was significantly reduced after exposure to 300 and 900 mg/kg DEHP. In part B, both PBP C3 and ODC were significantly reduced at 30 and 100 mg/kg.

Discussion

Our studies show that perinatal exposure to low doses of DEHP induces anti-androgenic effects such as reduced AGD, increased NR, weight reduction of LABC and mild external genitalia dysgenesis in male rats. Higher doses of DEHP, i.e. from 100 mg/kg, additionally induced histopathological effects in the testes (in part A), reduced testicular and prostate weight and reduced expression of androgen-regulated genes in the prostate.

Testicular histopathological effects following perinatal DEHP exposure have been reported in several studies. A dose-dependent reduction in testis weight, an increased number of testicular histological alterations, and absence of spermatocytes were reported in male offspring at 21 and 28 days of age in dams exposed to DEHP during gestation and lactation via the drinking water at estimated doses of 3.5 and 35 mg/kg [12]. In another study, small or aplastic testes and seminiferous tubular atrophy in male offspring was found at a dose level of 300 ppm in the diet (corresponding to approximately 14 mg/kg), while higher doses also caused histopathological changes in the epididymides and deceased weights of several male reproductive organs [15].

In the current study, testis changes were further characterized by measurement of seminiferous tubular diameter and immunohistochemical staining for the structural protein vimentin present in Sertoli cells. In the highest dose groups, Sertoli cells appeared to be affected by DEHP treatment, as vimentin staining was more intense and extended more diffusely throughout the cytoplasm of the Sertoli cells, and tubular diameter and testis weight were reduced. Sertoli cell number is known to relate closely to testis size [32]. Another marker of Sertoli cell number and function is the serum inhibin B level, which is altered with experimental manipulation of Sertoli cell number [33]. In our previous studies with higher doses (750 mg/kg bw) of DEHP, serum inhibin B levels were reduced at PND 22, but in the

current study no changes were seen at PND 16. This indicates that the effects on inhibin B and thereby on Sertoli cell number and/or function have not emerged at PND16 but rather initiates at a later time point.

The expression levels of androgen-regulated genes were sensitive markers of anti-androgenic effects on the prostate. PBP C3 and ODC contain an androgen-responsive-element in the promoter region and their expression is thereby influenced by the amount of androgen or anti-androgen available. Transcript levels of both PBPC3 and ODC were reduced with 30 mg/kg DEHP in part B. In part A, reductions were seen from 100 mg/kg but were not statistically significant, possibly due to small group sizes and large variations within groups. This supports the anti-androgenic effects of DEHP and may be secondary to decreased testosterone formation or the AR antagonistic effect of metabolites of DEHP. Reduced ODC and PBP C3 expression levels have previously been reported in ventral prostates after exposure to AR antagonists such as vinclozolin, fenarimol and prochloraz [30,34,35], but this is the first paper to describe alterations after perinatal DEHP exposure.

Anti-androgenic effects of DEHP similar to those described here have been demonstrated in numerous rat studies using dose levels above 100 mg/kg. Gray et al. [7] found effects on AGD, NR and reproductive organ weights at 750 mg/kg. Jarfelt et al. [8] found that DEHP decreased AGD and increased retention of nipples in male offspring at 300 and 750 mg/kg. In the same study, dosed males also showed decreased weights of ventral prostate and LABC, and histopathological investigations revealed alterations in testis morphology in both juvenile and adult males. In a two-generation study by Wolfe and Layton, incomplete masculinization and increased sensitivity was observed in male pups dosed perinatally, as the F1 and F2 generations showed more severe effects than the parental generation who were

exposed only as adults. DEHP caused decreased weight of male reproductive organs such as the testes, epididymides, and the prostate, reduced AGD and increased NR [15].

A more limited number of studies have investigated effects of perinatal dose levels below 100 mg/kg. Andrade and co-workers [36] looked at effects on aromatase activity in pups prenatally exposed to DEHP. The doses were 0.015, 0.045, 0.135, 0.405, 1.215, 15, 45, and 405 mg/kg/day. Aromatase activity was significantly inhibited at PND 1 at the low doses (0.135 and 0.405mg/kg), while an increased activity was observed at 15, 45 and 405 mg/kg. In the same study a reduction in daily sperm production was observed in animals exposed to 15, 45, 135 and 405 mg/kg/day and a low incidence of cryptorchidism was observed also in the 5 mg/kg dose group [11]. Effects on testes have been reported at a dose level of 300 ppm in the diet (about 14 mg/kg) and around 3.5 and 35 mg/kg via drinking water [12,15]. These results were included in an EU Risk Assessment Report of DEHP and resulted in a regulatory NOAEL (No Observed Adverse Effect Level) of 5 mg/kg for developmental toxicity [37]. In November 2005 US CERHR (Center For The Evaluation of Risk To Human Reproduction) published an update on the reproductive and developmental toxicity of DEHP, in which a NOAEL between 1 and 10 mg/kg for oral DEHP exposure in rats was supported [38].

Our results extend the database for low dose effects of DEHP on male sexual differentiation, as the two studies included low dose levels of DEHP, i.e. 3, 10 and 30 mg/kg.

The results from the two studies presented here are not identical, as for example significantly decreased AGD and increased NR were found in part A at 10 mg/kg and higher, while AGD only differed significantly from control values at 100 mg/kg in part B. Also, the incidence of male offspring with mild external genital dysgenesis was significantly different from control at 100 mg/kg and higher in part A, while in part B significant effects were found at 3 and 100

mg/kg. Effects on gene expression were found after exposure to 30 mg/kg in part B, while in part A, no statistically significant effect was observed until dose 300 mg/kg, although a tendency was already present at 100 mg/kg. This indicates that animals in part B were more affected at this endpoint compared to the animals in part A. These discrepancies between the two parts are surprising, but could be due to the relatively low number of animals per group and the biological variation in the investigated parameters. The postimplantation – perinatal loss at 10 mg/kg (in part B) should be discussed because the low number of animals may contribute substantially to the discrepancies between part A and B. Therefore, an important consideration when performing low dose studies is to include sufficient number of animals per group to avoid false-positive or false-negative results.

Our combined analysis of the data from the two studies is based on a more sufficient group size of 16 litters and therefore provides a more clear and reliable picture of the anti-androgenic effects of DEHP. The combined results show that perinatal exposure to low doses of DEHP induces anti-androgenic effects such as reduced AGD, increased NR, weight reduction of LABC and mild external dysgenesis in male rats at 10 mg/kg

Some indications of anti-androgenic effects were also found at the lowest dose of 3 mg/kg, as the incidence of male offspring having mild external genital dysgenesis and more than 2 nipples were increased. These results indicate that although this dose level did not induce significant effects on AGD, mean number of nipples or reproductive organ weights, subtle anti-androgenic effects may occur. Further studies, however, will be needed for a clear evaluation of the potential anti-androgenic effects of DEHP at 3 mg/kg and lower doses.

As mentioned in the introduction, DEHP is not a clear AR receptor antagonist like the 3 chemicals vinclozolin, procymidone and flutamide [39]. These AR receptor antagonists showed monotonous dose response curves on the same endpoints as investigated in the

current study. Our DEHP results seemed to show non-monotonous dose response curves at the low doses in relation to NR, incidence of male offspring with mild external malformations, and weight of LABC, with more pronounced effects at 10 mg/kg. These endpoints are all measured during the later part of the lactation period. The unusual dose response curves therefore might be due to special mechanisms or toxicokinetics during this period. However, biological variation for these endpoints may also be part of the explanation. Gee et al. found a non-monotonous (biphasic) effect on Leydig cell function when exposing male rats to DEHP in the prepubertal period (PND21-48) [36]. Andrade et al also found a non- monotonous dose response profile, a J-shaped curve while the aromatase activity was inhibited at low doses and increased at high doses in DEHP exposed (GD6-PND21) males PND1 [36,40].

In summary, our results demonstrate that DEHP at a relatively low dose of 10 mg/kg causes adverse anti-androgenic effects on male rat development. At this dose level, male AGD was decreased, the incidence of NR was increased, reproductive organ weight was reduced and mild external genitalia dysgenesis was observed. The results provide new evidence of low-dose effects of DEHP, supporting that the cautious NOAEL of 5 mg/kg for DEHP that has been decided in EU is appropriate. Our results also indicate a reason for concern about the current exposure level of the human population.

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Tables

Table 1. The number of litters in part A and part B is shown here and in parenthesis the number of mated animals is given.

DEHP (mg/kg bw/day)	**Control**	**3**	**10**	**30**	**100**	**300**	**600**	**900**
Part A	15 (16)	-	8 (8)	7 (8)	7 (8)	7 (8)	6 (8)	7 (8)
Part B	15 (16)	14 (16)	6 (8)	6 (8)	8 (8)	-	-	-
Total	30	14	14	13	15	7	6	7

Table 2a. Pregnancy and litter data from part A

	DEHP (mg/kg bw/day)						
	Control	**10**	**30**	**100**	**300**	**600**	**900**
Number of litters	15	8	7	7	7	6	7
Maternal weight gain GD7-21 (g)	86.2 ± 5.4	77.1 ± 6.1	93.4 ± 6.7	87.6 ± 9.6	80.4 ± 9.0	97.5 ± 9.3	74.3 ± 8.2
Maternal weight PND 1 (g)	235.5 ± 6.7	228.1 ± 5.1	237.0 ± 5.2	245.3 ± 8.8	234.1 ± 8.7	233.8 ± 7.5	224.9 ± 6.0
Pregnancy length (days)	22.7 ± 0.1	22.8 ± 0.2	22.7 ± 0.2	22.4 ± 0.2	22.7 ± 0.2	22.7 ± 0.2	22.4 ± 0.2
Birth weight (g)							
– Males	6.47 ± 0.16	6.63 ± 0.13	6.49 ± 0.06	6.40 ± 0.21	5.91 ± 0.15*	5.61 ± 0.26**	5.70 ± 0.20**
– Females	6.12 ± 0.13	6.09 ± 0.11	6.14 ± 0.11	5.83 ± 0.27*	5.76 ± 0.18	5.75 ± 0.13	5.29 ± 0.28**
Male pups (%)	53 ± 7	53 ± 7	41 ± 8	45 ± 5	34 ± 7	46 ± 7	52 ± 8
Live born per litter	9.6 ± 1.0	8.0 ± 1.2	10.3 ± 1.2	8.4 ± 1.1	9.4 ± 1.4	10.2 ± 1.0	8.4 ± 1.3
Postimplantation -perinatal loss (%) #	17.3 ± 7.0	20.9 ± 6.1	9.2 ± 3.6	14.1 ± 3.5	20.8 ± 11.0	18.1 ± 3.5	30.7 ± 12.2
AGD in males, at birth (mm)	3.68 ± 0.11	3.37 ± 0.11**	3.35 ± 0.12**	3.38 ± 0.12**	3.37 ± 0.12**	3.26 ± 0.12**	3.18 ± 0.12**
Body weight. day 12 ±1 (g)							
– Males	27.75 ± 1.0	29.27 ± 1.3	27.08 ± 1.4	31.38 ± 1.3*	24.23 ± 0.9	24.35 ± 0.8	23.38 ± 1.5*
– Females	26.43 ± 0.9	28.4 ± 1.4	25.64 ± 1.4	29.53 ± 1.1	24.74 ± 1.2	24.22 ± 0.8	23.39 ± 1.5
Nipples in males, day 12 ±1 (number)	0.22 ± 0.08	3.14 ± 0.94**	1.81 ± 0.82**	1.23 ± 0.68**	5.21 ± 1.25**	4.63 ± 1.72**	5.01 ± 1.36**

Data represent group means ± SEM

(No. implantations - live pups at PND 6)/no. implantations

The AGD values are corrected for birth weight and LSmeans values are presented.

* Indicates a value differs from control by $p < 0.05$

** Indicates a value differs from control by $p < 0.01$

Table 2b. Pregnancy and litter data from part B

	DEHP (mg/kg bw/day)				
	Control	**3**	**10**	**30**	**100**
Number of litters	15	14	6	6	8
Maternal weight gain GD7-21 (g)	79.6 ± 4.6	84.2 ± 4.4	79.7 ± 9.3	84.5 ±6.6	81.1±5.1
Maternal weight PND 1 (g)	249.9 ± 5.1	244.0 ± 5.0	250.4 ± 14.6	251.2 ± 6.3	244.3 ± 5.4
Pregnancy length (days)	22.5 ± 0.1	22.5 ± 0.1	22.5 ± 0.2	22.3 ± 0.2	22.5 ± 0.2
Birth weight, (g)					
– Males	6.25 ± 0.12	6.22 ± 0.10	6.14 ± 0.14	5.88 ± 0.19	5.87 ± 0.23
– Females	6.00 ± 0.16	5.92 ± 0.12	5.88 ± 0.14	5.55 ± 0.15	5.48 ± 0.23
Male pups (%)	47 ± 4	43 ± 4	38 ± 9	51 ± 4	44 ± 4
Live born per litter	9.4 ± 0.8	10.9 ± 0.7	8.1 ± 2.0	10.5 ± 1.1	11.3 ± 1.1
Postimplantation-perinatal loss (%) #	19.3 ± 3.6	9.8 ± 2.5	37.0 ± 13.5*	17.0 ± 4.7	14.8 ± 3.4
AGD in males, at birth (mm)	3.40 ± 0.07	3.36 ± 0.07	3.36 ± 0.08	3.38 ± 0.08	3.25 ± 0.07 *
Body weight, day 12 ±1 (g)					
– Males	30.56 ± 1.0	29.31 ± 0.7	30.97 ± 1.5	28.54 ± 1.7	28.93 ± 1.9
– Females	30.3 ± 1.0	28.71 ± 0.8	29.95 ± 1.5	28.45 ± 2.0	27.94 ± 1.8
Nipples in males, day 12 ±1 (number)	0.38 ± 0.92	0.59 ± 0.99	1.13 ± 1.26	0.31 ± 0.40	0.86 ± 1.23

Data represent group means ± SEM
(No. implantations - live pups at PND 6)/no. implantations
The AGD values are corrected for birth weight and LSmeans values are presented.
* Indicates a value differs from control by $p < 0.05$
** Indicates a value differs from control by $p < 0.01$

Table 3. Incidence of litters and offspring with nipple retention > 2 in males

DEHP, mg/kg bw/day	**Control**	**3**	**10**	**30**	**100**	**300**	**600**	**900**
Litters								
Study 1	0% (0/15)		50% (4/8)**	50% (3/6)*	14% (1/7)	83%** (5/6)	50%* (3/6)	83%** 5/6)
Study 2	7% (1/15)	14% (2/14)	17% (1/6)	0% (0/6)	13% (1/8)			
Study 1+2	3% (1/30)	14% (2/14)	36%(5/14)**	25% (3/12)	14% (2/15)			
Offspring								
Study 1	2% (1/62)		68%(19/28)**	31%(8/26)**	26%(6/23)**	77%(26/34)**	70%(16/23)**	65%(17/26)**
Study 2	5% (3/56)	15% (9/59)	24% (5/21)	0% (0/26)	16% (5/32)			
Study 1+2	3% (4/118)	15%(9/59)*	49%(24/49)**	15%(8/52)*	20%(11/55)*			

Data shown are percent affected (affected/total)

* Statistical significant different compared to controls ($p < 0.05$)

** Statistical significant different compared to controls ($p < 0.01$)

Table 4. Incidence of male offspring with mild external genital dysgenesis

	DEHP mg/kg bw/day							
	Control	**3**	**10**	**30**	**100**	**300**	**600**	**900**
Affected males of total males study 1	2% (1/48)		14% (4/28)	4% (1/26)	17% (4/23)*	17% (4/23)*	17% (4/23)*	50% (13/26)*
Affected males of total males study 2	0% (0/37)	12% (6/49)*	10% (2/26)	8% (2/26)	15% (3/20)*			
Affected males of total males study 1 and 2	0.1%(1/85)		11% (6/54)*	6% (3/52)	16% (7/43)**			
Affected litters of total litters study 1	7% (1/15)		38% (3/8)	14% (1/7)	57% (4/7)*	43% (3/7)	50% (3/6)*	67% (4/6)*
Affected litters of total litters study 2	0% (0/15)	29% (4/14)*	17% (1/6)	33% (2/6)	25% (2/8)			
Affected litters of total litters study 1and 2	3%(1/30)		29% (4/14)*	23% (3/13)	40% (6/15)**			

Analysed by a Fisher's exact test

* Statistical significant different compared to controls ($p < 0.05$)

** Statistical significant different compared to controls ($p < 0.01$)

Table 5. Body and organ weights for DEHP-exposed male pups at PND 16

Study 1	**Control**	**3mg**	**10 mg**	**30 mg**	**100 mg**	**300 mg**	**600 mg**	**900 mg**
Body weights (g)	34 ± 1		38 ± 1	33 ± 2	38 ± 2*	30 ± 2*	30 ± 2*	29 ± 2*
Right testis (mg)[§]	63.8 ± 1.2		63.0 ± 1.6	62.7 ± 1.6	58.8 ± 1.7*	60.5 ± 1.7	59.1 ± 1.7*	54.7 ± 1.8**
Left testis (mg)[§]	63.5 ± 1.5		63.5 ± 2.0	63.7 ± 2.1	59.4 ± 2.2	60.6 ± 2.1	56.5 ± 2.2**	55.8 ± 2.2**
Epididymides (mg)[§]	24.7 ± 1.0		24.0 ± 1.3	21.4 ± 1.4	22.2 ± 1.4	22.5 ± 1.4	22.4 ± 1.4	22.0 ± 1.5
Ventral prostate (mg)[§]	15.1 ± 0.6		13.6 ± 0.8	13.2 ± 0.9*	13.0 ± 0.9*	12.5 ± 0.9*	12.2 ± 0.9**	11.1 ± 0.9**
Seminal vesicles (mg)	14.4 ± 1.0		14.4 ± 1.5	14.5 ± 1.7	12.7 ± 1.6	18.7 ± 1.6	15.4 ± 1.6	15.2 ± 1.7
LABC[#] (mg)[§]	23.7 ± 1.1		19.1 ± 1.3**	20.0 ± 1.4*	20.1 ± 1.4*	20.0 ± 1.5*	21.0 ± 1.4	19.6 ± 1.5*
Bulbourethral glands (mg)	1.8 ± 0.1		1.6 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	2.0 ± 0.2
Adrenals (mg)[§]	10.0 ±0.3		8.8 ± 0.4**	9.4 ± 0.4	8.8 ± 0.4*	9.5 ± 0.4	9.3 ± 0.4	8.8 ± 0.4*
Kidneys (mg)[§]	348 ± 32		410 ± 44	425 ± 46	336 ± 50	344 ± 52	357 ± 51	351 ± 55
Liver (mg)[§]	884 ± 8.1		89 8 ± 12	876 ± 12	905 ± 13	922 ± 13*	916 ± 12*	955 ± 13**
Diameter, sem.tub.[¤](µm)	91.6 ± 1.7		92.2 ± 2.4	88.2 ± 2.6	87.2 ± 2.5	85.1 ± 2.5*	85.0 ± 2.6*	82.8 ± 2.6*
Study 2								
Body weights (g)	38 ± 1	36 ± 1	38 ± 2	35 ± 2	36 ± 2			
Right testis (mg)[§]	64.5 ± 1.3	67.3 ± 1.2	65.7 ± 1.9	64.7 ± 1.8	66.2 ± 1.7			
Left testis (mg)[§]	64.9 ± 1.5	67.0 ± 1.4	65.1 ± 2.2	63.9 ± 2.1	67.6 ± 1.9			
epididymides (mg)	25.0 ± 1.6	24.3 ± 1.6	23.4 ± 1.8	22.4 ± 1.9	25.7 ± 1.8			
Ventral prostate (mg)[§]	17.1± 0.9	16.7 ± 0.9	15.2 ± 1.3	14.6 ± 1.3	17.6 ± 1.1			
Seminal vesicles (mg)	13.4 ± 0.9	12.6 ± 1.0	11.3 ± 1.5	11.1 ± 1.5	12.1 ± 1.3			
LABC[#] (mg)	24.7 ± 1.3	23.4 ± 1.3	17.6 ± 1.8**	20.1 ± 1.8*	22.1 ± 1.6			
Bulbourethral glands (mg)	2.0 ± 0.1	1.9 ± 0.2	1.6 ± 0.2	1.8 ± 0.2	1.8 ± 0.2			
Adrenals (mg)[§]	10.3 ± 0.4	10.1 ± 0.4	9.5 ± 0.6	10.2 ± 0.6	10.4 ± 0.5			
Kidneys (mg)[§]	383 ± 5.5	380 ± 5.8	380 ± 8.5	380 ±8.6	379 ± 7.4			
Liver (mg)[§]	981 ± 12	958 ± 13	999 ± 19	949 ± 19	974 ± 16			
Diameter, sem.tub.[¤](µm)	89.4 ± 2.8	88.2 ± 2.6	90.4 ± 3.0	85.1 ± 3.0	88.7 ± 2.6			
Study 1 and 2								
Body weights (g)	36 ± 1		38 ± 1	34 ± 1	37 ± 1			
Epididymides (mg)[§]	25.1 ± 0.94		24.3 ± 1.1	22.1 ± 1.2	24.5 ± 1.1			
Ventral prostate (mg)[§]	16.5 ± 0.5		14.9 ± 0.7	14.2 ± 0.8	15.5 ± 0.7			
LABC[#] (mg)	24.4 ± 0.7		18.8 ± 1.0**	20.3 ± 1.1**	21.6 ± 1.0*			
Bulbourethral glands (mg)	1.9 ± 0.1		1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1			
Adrenals (mg)[§]	10.5 ± 0.2		9.4 ± 0.3	10.0 ± 0.4	9.7 ± 0.3			
Kidneys (mg)[§]	376 ± 3.8		370 ± 5.5	372 ± 6.0	372 ± 5.3			
Diameter, sem.tub.[¤](µm)	91.0 ± 1.5		91.4 ± 1.9	87.0 ± 1.9	87.9 ± 1.8			

Data represent least squares means ± SEM. The combined data for study 1 and 2 are shown only for endpoints for which the factor 'study' was not significant in the statistical analysis.

* Statistical significant different compared to controls ($p < 0.05$)

** Statistical significant different compared to controls ($p < 0.01$)

[#] Seminiferous tubules abbreviated sem.tub.

[¤] levator ani/bulbocavernosus muscles abbreviated LABC

[§] Statistical significant effect of the covariate, body weight ($p < 0.05$)

Figure legends

Figure 1
Mean anogenital distance (AGD) measured on PND 1 in male rat offspring of dams administered corn oil (control), 3, 10, 30 or 100 mg/kg bw/day DEHP from GD 7 to PND 16. Least square means + SEM are shown and the data are corrected for body weight and litter effect. Data represent the combined analysis of part A and B. ** Indicates $p < 0.01$. Gestational exposure to DEHP significantly reduces AGD in male rat offspring at 10, 30, and 100 mg/kg bw/day.

Figure 2
Mean number of nipples (NR) in male rat offspring of dams exposed to corn oil (control), 3, 10, 30 or 100 mg/kg bw/day DEHP from GD 7 to PND 16. Results are based on analysis of litter means and are presented as mean + SEM. Data represents the combined analysis of part A and B. * Indicates $p \leq 0.05$, ** indicates $p < 0.01$.The exposure statistically significantly increased NR in male rat offspring at 10, 30, and 100 mg/kg bw/day.

Figure 3
Testicular histopathology at PND 16 in rats exposed to vehicle (A, C), or 900 mg DEHP/kg bw/day (B, D) from GD 7 to PND 16. A and B: H&E staining; C and D: immunohistochemical staining for vimentin. In DEHP-exposed offspring, testes appeared immature with a reduced number of germ cells and a reduced seminiferous tubule diameter compared to controls (A, B). Focal Leydig cell hyperplasia was observed (#). Vimentin staining showed a more intense staining in Sertoli cells of DEHP exposed animals compared to controls (C, D).

Figure 4
Expression of PBPC3 and ODC mRNA in ventral prostate relative to the expression of the house-keeping gene 18SrRNA determined by real-time (RT) PCR after DEHP exposure (PND16). Part A: 0, 10, 30, 100, 300, 600 and 900 mg/kg bw/day DEHP (n=5 to 8). Part B: 0, 3, 10, 30 and 100 mg/kg bw/day DEHP (n=4 to 12). Results are shown in means ± SEM * Indicates $p < 0.05$; ** Indicates $p < 0.01$ compared to control group

Figures

Figure 1

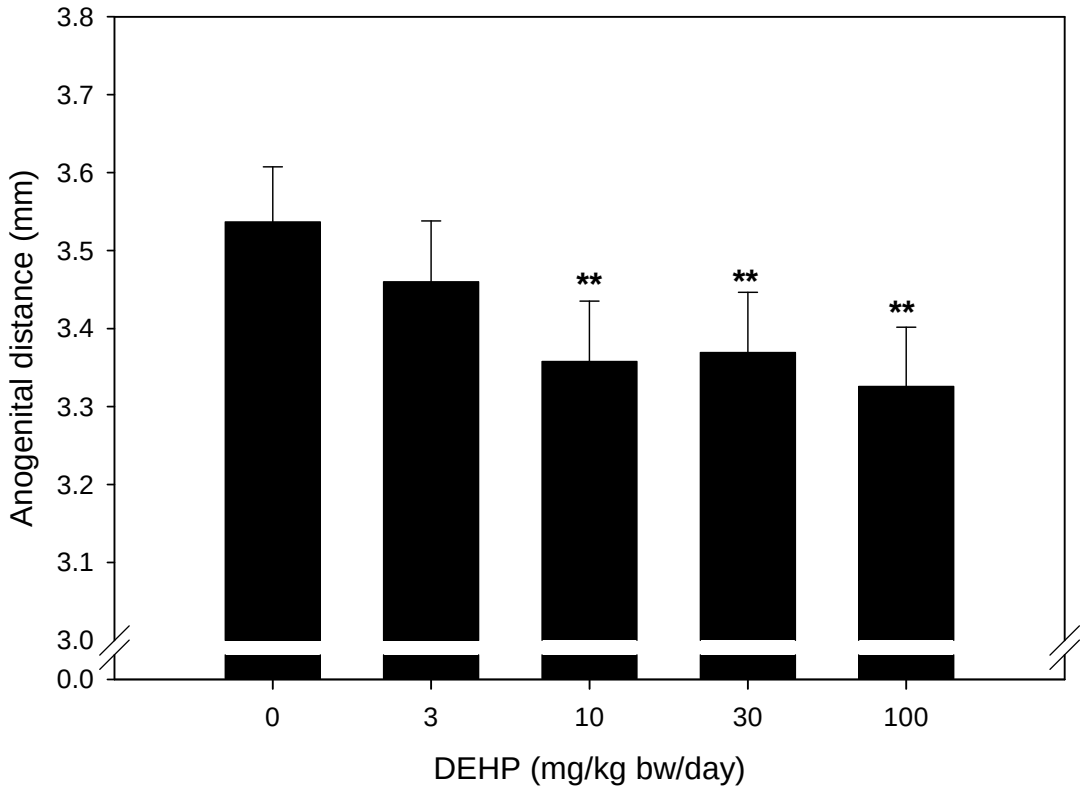


Figure 2

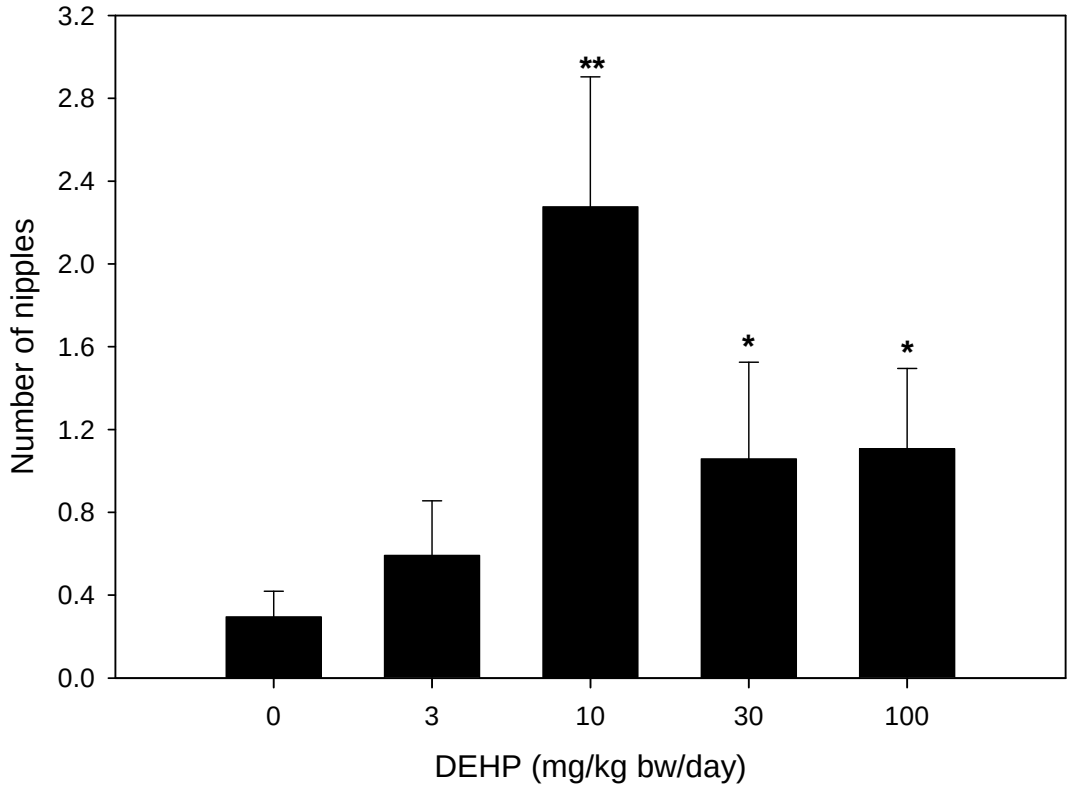

3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Number of nipples
**
*
*
0
3
10
30
100
DEHP (mg/kg bw/day)

Figure 3A-D

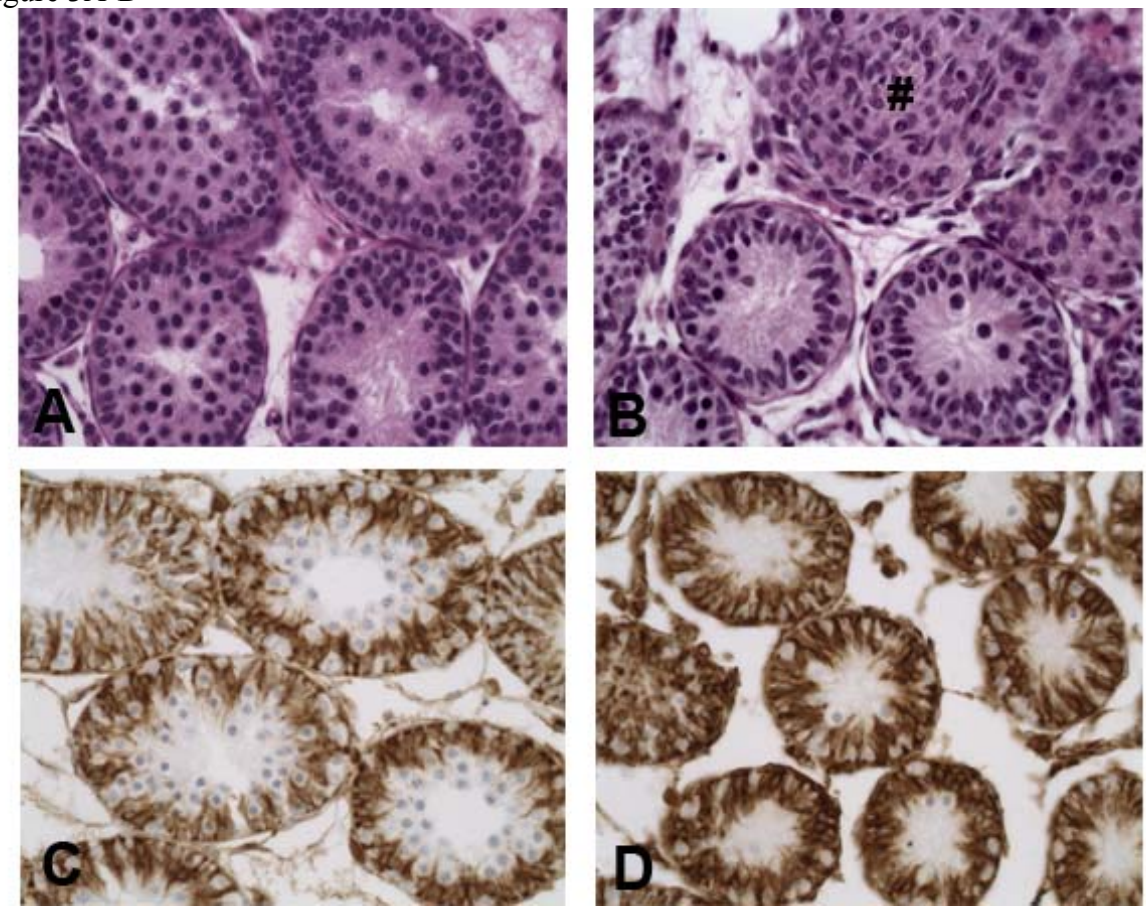
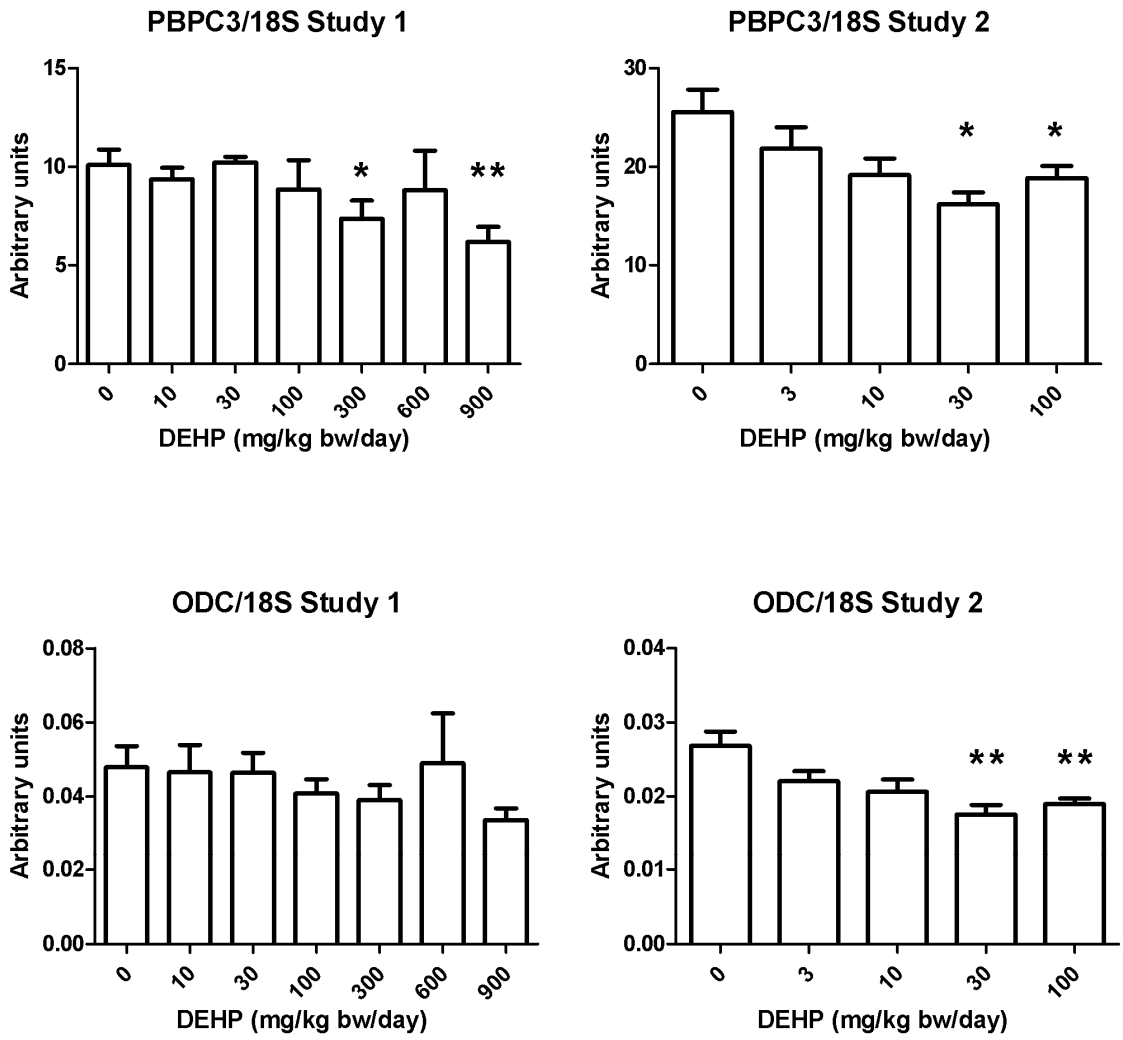


Figure 4


PBPC3/18S Study 1
Arbitrary units
15
10
5
0
*
**
0
10
30
100
300
600
900
DEHP (mg/kg bw/day)
PBPC3/18S Study 2
Arbitrary units
30
20
10
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0
3
10
30
100
DEHP (mg/kg bw/day)
ODC/18S Study 1
Arbitrary units
0.08
0.06
0.04
0.02
0.00
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DEHP (mg/kg bw/day)
ODC/18S Study 2
Arbitrary units
0.04
0.03
0.02
0.01
0.00
**
**
0
3
10
30
100
DEHP (mg/kg bw/day)

Paper II

Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, Metzdorff SB, Kortenkamp A. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. Environmental Health Perspectives 15:122-128 (2007).

Combined Exposure to Anti-Androgens Exacerbates Disruption of Sexual Differentiation in the Rat

Ulla Hass,[1] Martin Scholze,[2] Sofie Christiansen,[1] Majken Dalgaard ,[1] Anne Marie Vinggaard,[1] Marta Axelstad,[1] Stine Broeng Metzdorff ,[1] and Andreas Kortenkamp[2]

[1]Danish Institute for Food and Veterinary Research, Department of Toxicology and Risk Assessment, Søborg, Denmark; [2]The School of Pharmacy, University of London, London, United Kingdom

Objective: The aim of this study was to assess whether the joint effects of three androgen receptor antagonists (vinclozolin, flutamide, procymidone) on male sexual differentiation after *in utero* and postnatal exposures can be predicted based on dose–response data of the individual chemicals.

Methods: Test chemicals and mixtures were administered by gavage to time-mated nulliparous, young adult Wistar rats from gestational day 7 to the day before expected birth, and from postnatal days 1–16. Changes in anogenital distance (AGD) and nipple retention (NR) in male offspring rats were chosen as end points for extensive dose–response studies. Vinclozolin, flutamide, and procymidone were combined at a mixture ratio proportional to their individual potencies for causing retention of six nipples in male offspring.

Results: With AGD as the end point, the joint effects of the three anti-androgens were essentially dose additive. The observed responses for NR were slightly higher than those expected on the basis of dose addition. A combination of doses of each chemical, which on its own did not produce statistically significant AGD alterations, induced half-maximal mixture effects. At individual doses associated with only modest effects on NR, the mixture induced NR approaching female values in the males.

Conclusions: Effects of a mixture of similarly acting anti-androgens can be predicted fairly accurately on the basis of the potency of the individual mixture components by using the dose addition concept. Exposure to anti-androgens, which individually appears to exert only small effects, may induce marked responses in concert with, possibly unrecognized, similarly acting chemicals.

Key words: AGD, anti-androgen, combination effect, developmental exposure, flutamide, mixture, nipple retention, procymidone, vinclozolin. *Environ Health Perspect* 115(suppl 1):122–128 (2007). doi:10.1289/ehp.9360 available via *http://dx.doi.org/* [Online 8 June 2007]

Androgens are key regulators of male sexual differentiation during the *in utero* and early postnatal development. Exposure to chemicals that counteract androgen action at some stage in this period can permanently demasculinize male fetuses and lead to malformations of the reproductive tract. Examples of chemicals known to disrupt sexual differentiation in this way include pesticides and their metabolites, such as vinclozolin, procymidone, 1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene (*p,p′*-DDE), and linuron, and certain phthalate esters such as di-ethylhexyl phthalate and di-butyl phthalate (Gray et al. 2000, 2001). Reduced anogenital distance, retention of nipples or areolas, hypospadias, agenesis of sex accessory tissues, and undescended testes have been described as consequences of disruption of androgen action in the developing rat. These effects are thought to arise through antagonism of androgens at the steroid receptor level and/or *via* suppression of testosterone synthesis in Leydig cells (Fisher 2004; Gray et al. 2001).

Many anti-androgenic chemicals have been found as mixtures in humans (Blount et al. 2000; Swan et al. 2005), including children (Brock et al. 2002; Main et al. 2006), and in wildlife (Guillette 2000). These findings have stimulated interest in exploring the consequences of combined exposures to anti-androgens, although relatively few studies have addressed the issue. There is good evidence that inhibition of androgen binding and other receptor-mediated events occur in an additive fashion (Birkhoj et al. 2004; Gray et al. 2001; Nellemann et al. 2003), but little is known about the developmental effects of *in utero* and early postnatal exposure to multiple anti-androgenic chemicals.

In this article, we present data from detailed investigations of the ability of combinations of androgen receptor (AR) antagonists to induce disruption of male sexual differentiation after long-term exposures *in utero* and postnatally. We selected a mixture of vinclozolin, procymidone, and flutamide for our experiments. Vinclozolin metabolites compete with androgens for AR binding (Kelce et al. 1994), suppress androgen-dependent gene transcription (Kelce et al. 1997), and affect reproductive development. Procymidone and flutamide also antagonize competitively the AR binding of androgens, with consequent inhibition of AR-mediated gene expression (Ostby et al. 1999; Simard et al. 1986). Common developmental effects of all three chemicals after *in utero* exposure of male rats include reduced anogenital distance (AGD), nipple retention (NR), hypospadia, diminished prostate weight, reduced testis and epididymal weights, and altered behavior in male offspring (Foster and McIntyre 2002; Gray et al. 1994; Hellwig et al. 2000; Hib and Ponzio 1995; Hotchkiss et al. 2002; McIntyre et al. 2001; Miyata et al. 2002; Ostby et al. 1999; Shimamura et al. 2002). There is no particular environmental relevance to this mixture. The choice of compounds was motivated by our interest to explore the predictability of combination effects caused by similarly acting anti-androgens rather than to emulate "real world" mixtures.

Conclusive answers to the question of combination effect predictability require quantitative comparisons between predicted and experimentally observed mixture effects. Experimentally, we have approached this task in a step-wise fashion: *a*) Dose–response curves for all single-mixture components were recorded. *b*) These data were used for the calculation of additivity expectations for a mixture of specific composition using "fixed mixture ratio design" (Altenburger et al. 2000; Hewlett and Plackett 1959). *c*) The mixture experiments were conducted. *d*) The observed combination effects were compared with the predicted responses.

The choice of an appropriate model for the calculation of additivity expectations is essential for assessments of mixture effects because it is in relation to these additivity expectations that combination effects are judged in terms of synergisms or antagonisms. Several concepts for the computation of expected additive effects of anti-androgens have been used. The simple method of summing the individual effects of chemicals in the combination, termed "effect summation," has been drawn on previously (Gray et al.

This article is part of the monograph "Endocrine Disruptors—Exposure Assessment, Novel End Points, and Low-Dose and Mixture Effects."

Address correspondence to U. Hass, Danish Institute for Food and Veterinary Research, Dept. of Toxicology and Risk Assessment, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark. Telephone: 45 72347544. Fax: 45 72347001. E-mail: ulh@dfvf.dk

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2001) but produces unreliable results with sigmoidal dose–response curves (Kortenkamp and Altenburger 1998). The concept of dose addition, also referred to as "concentration addition" (Loewe and Muischnek 1926), is usually employed for combinations of chemicals with similar modes of action. It has previously given additivity expectations well in agreement with experimental observations for inhibition of AR binding and AR-mediated responses *in vitro* and *in vivo* (Birkhoj et al. 2004; Nellemann et al. 2003). In light of these observations, we reasoned that dose addition would also produce valid additivity expectations for developmental effects after prolonged *in utero* and postnatal exposures.

Although a series of articles have been published describing the successful application of the fixed mixture ratio approach to *in vitro* systems (Altenburger et al. 2000; Backhaus et al. 2000; Payne et al. 2001; Rajapakse et al. 2002, 2004; Silva et al. 2002), there is comparatively little experience with *in vivo* assays. In the endocrine disruptor field, Brian et al. (2005) have recently demonstrated the usefulness of this method to the assessment of multicomponent mixtures of estrogenic chemicals in fish, but there are as yet no examples with mammalian assays *in vivo*. Thus, to make the assessment of developmental effects of mixtures of chemicals a viable proposition, a number of practical requirements had to be considered. Of particular importance were demands of minimal data variation and high reproducibility. When dealing with several mixture components and a large number of dose levels, the parallel testing of all agents and their mixtures is not a realistic option, especially not with *in vivo* experiments. Thus, reliance had to be made on historical data, in some cases recorded more than a year before commencement of the mixture experiments, and this placed great emphasis on the reproducibility of test outcomes. We considered that the high demands in terms of data variation were more likely to be met with developmental end points that lend themselves to straight-forward quantification. For these reasons, we selected changes in AGD and NR in male offspring of rats as main end points for our mixture experiments. Both these end points are sensitive to anti-androgen exposure.

The aim of our studies was to assess whether the joint effects of mixtures of AR antagonists can be predicted accurately over a large effect range on the basis of dose–response data of the individual components. We reasoned that if there are demonstrable consistent relationships between the potency of individual chemicals and the ways in which they act together, powerful tools for prospective risk assessment would become available. These tools could open the way to make productive use of existing single-chemical databases for the prediction of mixture effects. A second aim was to determine whether there would be joint effects when every mixture component was present at doses that individually do not produce observable responses.

Materials and Methods

Chemicals. The chemicals used were vinclozolin (CAS No. 50471-44-8, purity 99%, ChemService catalogue no. PS-1049; Bie & Berntsen, Herlev, Denmark), procymidone (CAS No. 32809-16-8, purity 99%, ChemService catalogue no. PS-2126; Bie & Berntsen), flutamide (CAS No. 13311-84-7, purity 99%, catalogue no. F9397; Sigma Aldrich, Brønby, Denmark), and corn oil used as vehicle (Bie & Berntsen).

Studies and dose levels. Before the mixture experiment, dose–response studies for each chemical were conducted. The dose ranges were chosen with the aim to cover the entire range of effects from no effect up to maximum effects, as determined by measurement of AGD and NR. At the same time, it was attempted to select doses that would not cause marked effects on body weights in the dams, and especially in the offspring, as this would complicate evaluation of the effects on AGD and NR. The dose levels selected for the dose–response studies were based on the reductions of AGD and increase of NR reported for vinclozolin (Gray et al. 1994, 1999; Hellwig et al. 2000; Hotchkiss et al. 2002; Shimamura et al. 2002), flutamide (Foster and McIntyre 2002; Hib and Ponzio 1995; Hotchkiss AK et al. 2002; McIntyre et al. 2001; Miyata et al. 2002), and procymidone (Ostby et al. 1999). As data on procymidone were relatively limited, a range-finding study was performed before the dose–response study. To gain information about variability of effects between studies, we ran selected doses of vinclozolin, flutamide, and procymidone in parallel with the mixture experiment. An overview of the studies including dose levels and number of animals is shown in Table 1. A similar study design was used for all studies (see below).

For the mixture study, a master mixture was prepared by combining doses of vinclozolin, flutamide, and procymidone that all induced a half-maximal degree of NR (six nipples) in male offspring. This approach was chosen to avoid one single chemical contributing disproportionately to the overall mixture effect. The resulting mixture ratio of vinclozolin, flutamide, and procymidone was 31:1:18 based on weight (Table 2), and the master mixture contained 22,026 mg vinclozolin, 696.6 mg flutamide, and 12,675 mg procymidon in 600 mL corn oil. It was diluted into five doses of the mixture (1:14, 1:5, 1:2, 6:4, and 9:1). These dose levels were chosen with the aim of covering the entire dose–response curve.

Animals and dosing. The animals were treated humanely and with regard for alleviation of suffering. The studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the inhouse Animal Welfare Committee.

Time-mated nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark; body weight approximately 200 g) were supplied at day 3 of pregnancy. The day after mating was designated gestational day (GD) 1, and postnatal day (PND) 0 was the day of birth. On the day after arrival (GD4), the dams were distributed pseudorandomly into groups of 16 or 8 animals with similar body weight (bw) distributions. They were housed in pairs until GD21 and alone thereafter under standard conditions in semitransparent plastic cages (15 × 27 × 43 cm) with Aspen bedding (Tapvei, Gentofte, Denmark) situated in an animal room with controlled environmental conditions (12-hr light–dark cycles with light starting at 2100 hours, light intensity 500 lux, temperature 21 ± 2°C, humidity 50% ± 5%, ventilation eight air changes per hour). A complete rodent diet for growing animals ALTROMIN 1314 (soy- and alfalfa-free; ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) were provided *ad libitum*.

Test chemicals and mixtures were administered by gavage from GD7 to the day before expected birth (GD21) and from

Table 1. Studies, groups, doses, and number of time-mated animals per group.

Study	Groups and doses	No. of animals per group
1. Vinclozolin and flutamide, dose–response	Control: vehicle-dosed	16
	6 doses of vinclozolin: 5, 10, 20, 40, 80, or 160 mg/kg/day	8
	6 doses of flutamide: 0.5, 1.0, 2.0, 4.0, 8.0, or 16 mg/kg/day	8
2. Procymidone, range-finding	Control: vehicle-dosed	16
	2 doses of procymidone: 25 or 200 mg/kg/day	4
3. Procymidone, dose–response	Control: vehicle-dosed	16
	6 doses of procymidone: 5, 10, 25, 50, 100, or 150 mg/kg/day	8
4. Mixture study of vinclozolin, flutamide, and procymidone	Control: vehicle-dosed	16
	5 doses of mixture: 7.87, 19.67, 39.33, 70.80, or 106.19 mg/kg/day	16
	2 doses of vinclozolin: 24.5 or 95.9 mg/kg/day	16
	2 doses of flutamide: 0.77 or 3.86 mg/kg/day	8
	2 doses of procymidone: 14.1 or 61.8 mg/kg/day	8

PND1 until PND16. The dosing volume of 2 mL/kg bw was calculated on the basis of the body weight of the animal on the day of dosing. The dose levels and group sizes are shown in Table 1. Animals were inspected for general toxicity twice daily. The studies were performed using four blocks (with 1 week in between), and all dose groups were equally represented in the blocks.

Anogenital distance and nipple retention. In all studies, AGD and NR were recorded by the same technician who was blinded with respect to exposure groups. After birth all live pups in the litter were weighed, sexed, and AGD was measured using a stereomicroscope. The sex of several of the pups in the highest dose groups could not be determined based on the AGD, as the AGDs were similar to female values in all pups in some litters. In these cases, the sex of the pups was determined later by internal inspection of reproductive organs with the presence of testes defining a male. The highest AGD values obtained in the litter were used as the values for male pups. This approach was chosen because these values were most likely to represent the males, as males normally have longer AGDs than females. For dose–response analysis, AGD data were analyzed by the calculated AGD-index, namely, AGD divided by the cube root of body weight. The cube root was used because this converts a three-dimensional end point (weight) into a one-dimensional such as the AGD (Gray et al. 1999; Robert et al. 1999). This ratio assumes that the relationship between AGD and transformed body weight is directly proportional and linear. To assess the validity of this assumption, we explored using the transformed body weight as a co-variable in statistical analyses. However, we did not detect any relevant difference between these two approaches, and in the interest of keeping the model parameters low, we decided to base all statistical analyses on the AGD index.

The body weights of all pups were recorded on PND 12 ± 1, together with the number of nipples/areolas, defined as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Females normally have 12 nipples, but may in a few cases show up to 14.

Data normalizations and dose–response analysis. Slight differences in absolute control values between studies were controlled for by standardizing the absolute AGD indices to relative values between one (no effect on male AGD index) and zero (complete feminization). The mean AGD indices from unexposed male and female pups were used to define the minimum and maximum responses, respectively. Regression analyses were based on normalized AGD indices. In contrast, absolute AGD indices were used for estimations of no observed adverse effect levels (NOAELs). Because NR is a quantal end point, data normalization was not necessary in this case.

Statistical dose–response regression analyses for both end points were carried out by applying a best-fit approach (Scholze et al. 2001). Various nonlinear regression models (logit, probit Weibull, generalized logit), which all describe monotonic sigmoidal dose-response relationships, were fitted independently to the same data set and the best-fitting model was selected on the basis of a statistical goodness-of-fit criterion, the information criterion of Schwarz (1978).

To control for litter effects on AGD, dose–response data for the normalized AGD indices were analyzed by a generalized nonlinear mixed-model approach (Vonesh and Chinchilli 1996), with the litter as a random effect modifier for individual AGD data.

To take account of uncertainties in the mean control estimates for AGD during the scaling of effects, we included an upper and lower asymptote in the regression models. However, neither for the individual compounds nor for the mixture were the resulting model parameters significantly different from 0 and 1, respectively. To avoid overparameterization, upper and lower asymptotes were therefore not estimated, but instead set *a priori* to 0 and 1 (see $\theta_{max} = 1$ in Table 2). Statistical analyses of AGD data were carried out using the SAS procedure PROC NLMIXED (SAS Institute Inc., Cary, NC, USA).

The number of nipples/areolas was assumed to follow a binomial distribution with a response range between 0 and θ_{max}, with θ_{max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13 (Table 2). The choice of θ_{max} was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipples/areolas and litter were modeled by the generalized estimating equations method (Vonesh and Chinchilli 1996). All statistical analysis was performed using the SAS procedure PROC GENMOD (SAS Institute, Inc., Cary, NC, USA).

The analyses for single compounds were carried out using effect data pooled from the initial dose–response studies (Table 1, studies 1–3) and the repeat experiments run concurrently with the mixture study (Table 1, study 4). "Study run" was implemented as an additional model factor in data analysis. The effect doses (ED_x) shown in Table 2 were selected for low and median response levels and were calculated from the functional inverse of the best-fitting model. Statistical uncertainties for the estimated effect doses were expressed as 95% confidence belts and approximately determined by applying the bootstrap method (Efron and Tibshirani 1993).

NOAELs were estimated using multiple contrast tests (Hothorn 2004). These tests were chosen as they are already implemented in the SAS procedures PROC NLMIXED and PROC GENMOD. Corresponding optimal contrasts were determined according to the best-fit regression model (Bretz et al. 2005).

Calculation of mixture–effect predictions using dose addition. To assess whether the joint effect of the three chemicals was dose additive, we predicted mixture effects based on information about the dose–effect relationships of all individual mixture components. These data were derived from the best-fit regression

Table 2. Statistical dose–effect descriptors for single and mixture exposures.

Substance	Fraction in mixture[b]	Dose–response function					Effect doses (mg/kg/day)		NOAEL[a] (mg/kg/day)
		RM[c]	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	$\hat{\theta}_{max}$	Medium	Low	
Nipple retention							ED_6[d] (95% CI)	ED_1[d] (95% CI)	
Vinclozolin	0.622301	BP-probit	–1.76	0.025	1.024	12	67.18 (55.78–77.08)	5.73 (0.67–25.58)	< 5.0
Flutamide	0.019558	Probit	–0.61	2.01	—	12	2.02 (1.71–2.40)	0.41 (0.28–0.60)	< 0.5
Procymidon	0.358141	BP-logit	–5.09	1.21	0.1	12	33.91 (24.81–45.74)	7.51 (> 0.1–10.76)	10.0
Mixture		bw-Weibull	–19.66	16.83	–0.8	13	20.78 (17.76–23.52)	8.21 (6.67–10.42)	< 7.87
AGD index							ED_{50}[e] (95% CI)	ED_{90}[e] (95% CI)	
Vinclozolin	0.622301	Logit	–6.80	3.59	—	1	78.65 (67.43–93.32)	9.21 (11.57–29.08)	5.0
Flutamide	0.019558	Weibull	–1.38	1.85	—	1	3.54 (2.84–4.37)	0.34 (0.19–0.57)	< 0.5
Procymidon	0.358141	bw-Weibull	–6.31	2.087	–0.20	1	69.38 (56.69–86.38)	1.84 (4.12–22.47)	10.0
Mixture		Glogit II	–9.24	7.21	0.29	1	39.77 (32.51–49.48)	4.68 (6.79–20.73)	19.67

[a]NOAEL – no observed adverse effect level, marked as "<" when the lowest tested dose already produced a significant effect. [b]Ratio of the dose of each compound to total mixture dose. [c]RM – regression models as defined by Scholze et al. (2001); for more details see "Material and Methods"; $\hat{\theta}_1$, $\hat{\theta}_3$, $\hat{\theta}_3$,– statistical estimates of model parameters, given for doses expressed as mg/kg/day (rounded values); $\hat{\theta}_{max}$– upper model asymptote. [d]ED_6, ED_1 – effect doses for 6 and 1 nipples, calculated from the respective dose–response function. [e]ED_{50}, ED_{90} – effect doses for 50% and 90% normalized AGD index, calculated from the respective dose–response function; 95% CI – 95% confidence intervals for mean effect doses given in mg/kg/day.

functions (Table 2) and used to calculate the expected responses of a mixture with defined mixture ratio over a large range of responses ("fixed mixture ratio design") (Faust et al. 2001). The choice of doses was based on the concentration range described by the additivity prediction, which is defined for a multicomponent mixture of three components as

$$EDx_{mixture} = \left(\frac{p_1}{EDx_1} + \frac{p_2}{EDx_2} + \frac{p_3}{EDx_3} \right)^{-1}. \quad [1]$$

Here, EDx_1, EDx_2, and EDx_3 are the effect doses of vinclozolin, flutamide, and procymidon that on their own produce the same quantitative effect x as the mixture, and p_1, p_2, and p_3 are the relative proportions of the corresponding individual doses present in the total mixture dose (see Table 2, "Fraction in mixture"). The individual effect doses were derived from the dose–response functions for vinclozolin, flutamide, and procymidone by using their inverse functional form. Equation 1 allows calculation of any effect dose of a mixture under the hypothesis of dose additivity, provided the dose–response functions of all mixture components and the mixture ratio are known. Graphs of predicted mixture dose–response curves (Figure 1) were obtained by calculating numerous $EDx_{mixture}$ values, with x varying from 10 to 90% for the normalized AGD index and from 1 to 11 for nipples. The statistical uncertainty for the predicted mixture–effect doses $EDx_{mixture}$ was determined by using the bootstrap method (Efron and Tibshirani 1993) and expressed as 95% confidence intervals (CIs) for the predicted mean estimate. Differences between predicted and observed effect doses were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

Results

Pregnancy and litter data. No clinical signs of general toxicity were observed during the daily observations. The maternal body weight gain from GD7 to PND1 was significantly decreased (12.8 ± 14.6 g compared with 24.3 ± 9.4 g in the control group) in dams receiving the highest dose of vinclozolin (160 mg/kg/day), but none of the other doses of vinclozolin provoked this effect. Pregnancy length, litter sizes, birth weight of male and female offspring, and sex ratios in the litters remained unaltered in all vinclozolin-dosed groups when compared to controls. None of the tested doses of flutamide induced reductions of maternal weight gain, or other signs of maternal toxicity. In the range-finding study with procymidone (Table 1, study 2), litter sizes were markedly decreased at the highest dose of 200 mg/kg/day. The dose–response study (Table 1, study 3) using 150 mg/kg/day as the highest dose did not show effects on pregnancy length, litter sizes, birth weights, or sex ratios in the litters. Maternal body weight gain from GD7 to PND1 was decreased in the dams exposed to 25 mg/kg/day procymidone and higher, but no clear dose–response relationship was apparent with these weight gain changes.

In dams exposed to the two highest doses of the mixture of vinclozolin, flutamide, and procymidone (Table 1, study 4), maternal body weight gain from GD7 to PND1 was decreased. Among the groups of pregnant rats that were dosed with the single agents in parallel with the mixture experiment (Table 1, study 4), those receiving the higher dose of procymidone (61.8 mg/kg/day) also had diminished weight gain. Decreased litter sizes were observed at the high dose of flutamide (3.86 mg/kg/day). As this was not found in the previous dose–response study at the similar dose level of 4 mg/kg/day, or at the higher doses of 8 and 16 mg/kg/day, this is considered a random finding unrelated to exposure to flutamide. None of the mixture doses caused significant effects on pregnancy length, litter sizes, birth weights, and sex ratios in the litters.

Effects of vinclozolin, flutamide, and procymidone on AGD and NR. All chemicals produced dose-dependent changes in AGD index and NR and the resulting dose–response curves were observed to be quite steep. The entire effect range from control levels to maximal responses could be covered by dose changes of only two orders of magnitude (Figure 2). While vinclozolin and procymidone were of similar potency, flutamide was effective at approximately 10-fold lower doses.

Compared with the AGD index, NR was generally the more sensitive end point. At the lowest tested doses of 5 and 0.5 mg/kg/day, respectively, vinclozolin and flutamide induced statistically significant changes in NR, whereas the respective AGD indices did not differ significantly from those of controls at these doses. NOAELs could therefore not be defined for vinclozolin and flutamide. For procymidone a NOAEL of 10 mg/kg/day was estimated.

To gain an impression of variability among studies, selected dose levels of all three chemicals were retested in parallel with the mixture experiment. The reproducibility of effects observed in the earlier studies (Table 1, studies 1–3) was generally good. At the lower doses, the animals in the repeat studies were slightly less responsive in terms of changes in AGD index, but the NR effects tended to be a little higher upon retesting (Figure 2; Table 3). Table 2 summarizes key parameters characterizing the dose–response relationships of each single substance. Generally, our data are in broad agreement with results published by others (Foster and McIntyre 2002; Gray et al. 1999; Hellwig et al. 2000; Hib and Ponzio 1995; Hotchkiss et al. 2002; McIntyre et al. 2001; Miyata et al. 2002; Ostby et al. 1999; Shimamura et al. 2002). However, because of the unprecedented level of detail in our dose–response analyses, more in-depth comparisons are not possible.

Combination effects of vinclozolin, flutamide, and procymidone. The mixture of vinclozolin, flutamide, and procymidone produced dose-dependent changes in AGD index and NR (Figure 1). The NOAEL for changes in AGD index was 19.67 mg/kg/day, but the lowest tested mixture dose of 7.87 mg/kg/day induced statistically significant changes in NR (Table 2). Therefore, the overall mixture NOAEL is lower than 7.87 mg/kg/day.

The dose–response data for the single agents, pooled from all studies (Figure 2; Table 2), were used to compute predicted dose-additive combination effects covering the entire range of effects (Figure 1, green curves). For both end points, the anticipated combination effects fell within the range of the effects that were observed experimentally.

Numerical comparisons between predicted and observed AGD index (Table 3) revealed fairly good agreement. Despite the long period that had elapsed between the recording of the effects of the individual mixture components

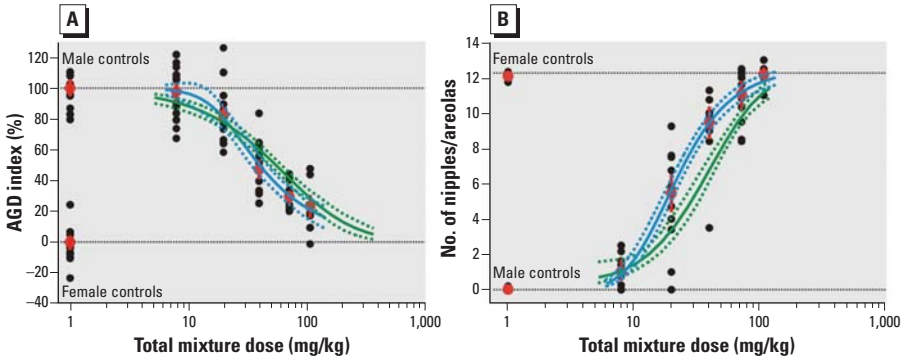

Figure 1. Effects of mixed exposure to vinclozolin, flutamide, and procymidone on AGD (*A*) and NR (*B*). Results shown are group mean ± SE (red), litter means (black), mean dose–response curve ± 95% confidence belt based on regression analysis (blue) and mean predicted mixture effect ± 95% confidence belt (green). Dashed horizontal lines show male and female control values. See text for details.

and the mixture experiment itself, the predicted effect doses in the median and high effect ranges differed by only a factor of 1.3 from those experimentally observed. Whether the anticipated combination effects were calculated using the data from the concurrent studies or using the pooled data sets including the historical data had little influence on the quality of the prediction. The joint effects of vinclozolin, flutamide, and procymidone on reductions of AGD in male rats were essentially dose additive.

In contrast, the deviations between prediction and observation were generally larger for NR than for AGD index, with observed NR responses exceeding the predicted mixture effects (Table 3; Figure 1). The effect doses predicted on the basis of the pooled single agent data were higher than the observed mixture–effect doses in the median- and high-effect range (6 and 10 retained nipples/areolas). This was not the case for the low-effect range (1 retained nipple/areola). Predictions based on the responses seen with single agents run in parallel with the mixture study (Table 1, study 4) produced lower effect doses in the median- and high-effect range, in better agreement with the observed results.

Mixture effects at low doses of individual mixture components. Because the doses of the single chemicals present in the mixture were quite low, we assessed whether there were significant combination effects when all components were present at doses that individually did not induce observable effects. At a dose of 39.37 mg/kg/day, the mixture induced a marked effect on the AGD index (around 50% reduction). This mixture contained 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone, and individually these doses did not induce significant reductions in the AGD index (Figure 3). With NR as the end point, the single-chemical effects were small but statistically significant at these doses, whereas the combined exposure induced a marked effect.

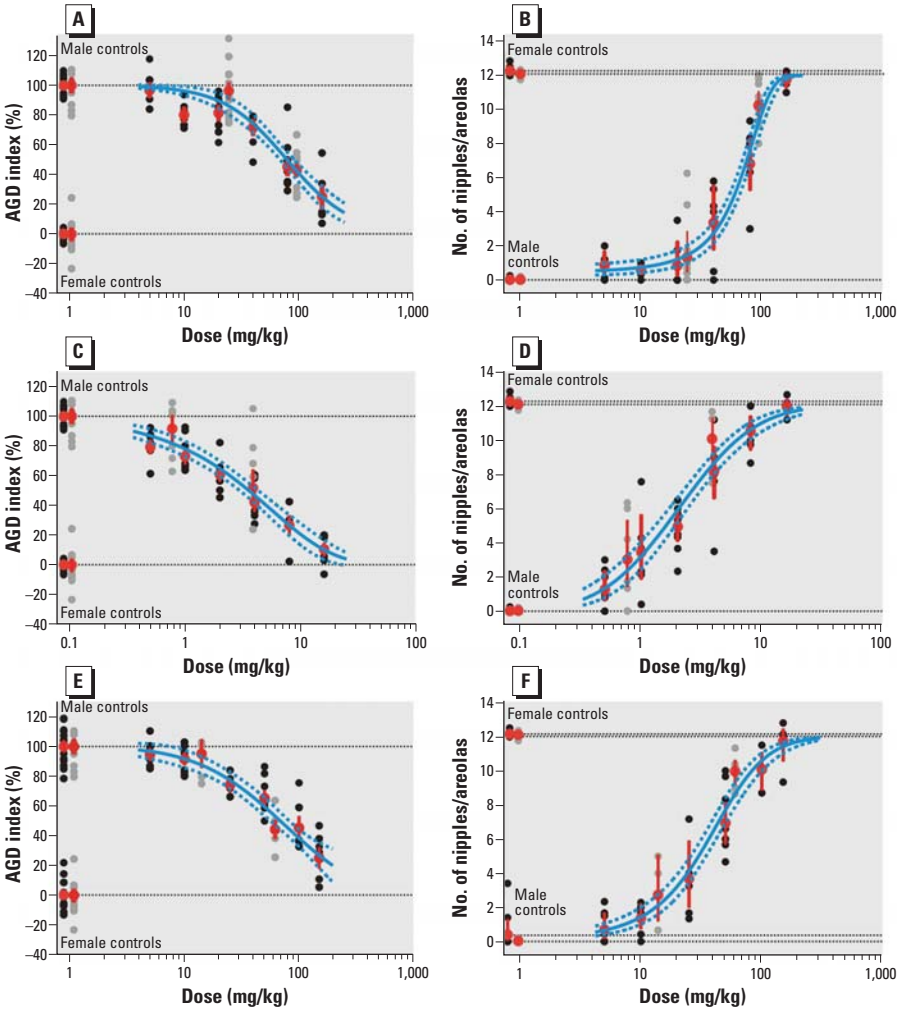

Figure 2. Effects of vinclozolin (*A,B*), flutamide (*C,D*), and procymidone (*E,F*), given individually, on AGD (left) and NR (right). Results shown are group mean ± SE (red), litter means from the dose–response studies (black), litter means from the single doses within mixture study (grey), and the mean dose–response curve ± 95% confidence belt based on regression analysis (blue). Dashed horizontal lines show male and female control values. See text for details.

Table 3. Statistical uncertainty of predicted and observed effect doses for the mixture of vinclozolin, flutamide, and procymidone.

	Effect doses for the mixture (mg/kg/day)		
Effect level	Observed[a] [Mean (95% CI)]	Predicted by DA_{pooled}[b] [Mean (95% CI)]	Predicted by $DA_{confirmation}$[c] [Mean (95% CI)]
Relative AGD index (%)			
90	14.68 (6.79–20.73)	8.29 (5.06–11.17)	14.98 (4.22–19.80)
50	39.77 (32.51–49.48)	53.75 (48.22–60.34)	52.58 (44.17–75.98)
20	112.45 (77.79–184.51)	143.19 (113.05–193.54)	129.34 (100.67–204.49)
Number of nipples/areolas			
1	8.21 (6.67–10.42)	7.43 (0.11–9.36)	7.45 (3.06–11.73)
6	20.78 (17.76–23.52)	33.88 (29.23–38.41)	27.98 (22.04–34.07)
10	45.33 (38.91–55.86)	73.66 (67.25–82.05)	57.31 (50.17–67.58)

[a]Effect doses as calculated from the dose–response functions given in Table 2. [b]DA, dose addition; predicted effect doses are based on pooled data from studies 1–4 for vinclozolin, flutamide, and procymidone and were calculated from the respective dose–response functions given in Table 2. [c]Predicted effect doses are based on data for vinclozolin, flutamide, and procymidone from study 4 only, where two doses each of these chemicals were run in parallel with the mixture experiment. Predicted mixture–effect doses were estimated by linear regression (model estimates not shown).

Discussion

Previous work with anti-androgens has focused mainly on events surrounding AR binding and activation and has shown that combinations of these chemicals are able to act together in an additive fashion (Birkhoj et al. 2004; Nellemann et al. 2003). These studies have prepared the ground for addressing the question as to whether there are also joint effects with responses further removed from receptor binding and activation, such as those related to male sexual differentiation. For the first time, we have addressed this question by using the fixed mixture ratio approach for studying combination effects on the disruption of male sexual development.

With reductions of AGD as the end point, the experimentally observed mixture effects were in good agreement with the dose additivity expectation calculated on the basis of the individual dose–response relationships for vinclozolin, flutamide, and procymidone.

Although the predicted mixture effects for NR also fell within the limits of the lowest experimentally recorded responses, the

NR responses in the median and high end indicated that the mixture was more potent than predicted. The movement away from the anticipated combination effects can be partly attributed to the fact that the single agent responses seen concurrently with the mixture study were slightly higher than previously recorded, particularly in the high-effect range. When the mixture–effect prediction was based solely on the data from the concurrently run single agent studies, the differences between anticipated and observed effect doses for NR became smaller. This could indicate that the animals used for the mixture experiment showed subtle differences in their responses to the anti-androgens compared with the rats used for the earlier dose–response studies. The reason such differences should have become apparent only in terms of altered NR, but not in relation to AGD, may lie partly in the greater sensitivity of NR as an anti-androgenic end point. However, other as yet unrecognized factors may also have played a role. Seen in this light, we hesitate to interpret the joint effects of the mixture on NR as weakly synergistic, although the numerical discrepancies between observed and anticipated additive effects would support such a conclusion. Much larger studies would be required to resolve conclusively whether vinclozolin, flutamide, and procymidone exhibit a weak synergism with respect to NR. Nevertheless, in view of the complexity of the events leading to alterations in AGD and NR, and considering the experimental challenges in recording such effects reliably and reproducibly over a long period, we were surprised that the combined effects of the three anti-androgens could be predicted quite accurately. We therefore conclude that the dose addition approach provides an excellent basis for prediction of the joint effects of multicomponent mixtures of similarly acting anti-androgens.

Although the primary aim of our work was to assess the predictability of mixture effects of anti-androgens, the results of our study also allow assessments of the question as to whether there are joint effects when all mixture components are present at doses that individually do not induce detectable effects. This phenomenon, somewhat provocatively dubbed "something from 'nothing'" (Silva et al. 2002), has been observed with multicomponent mixtures of estrogenic agents in reporter-based assays (Rajapakse et al. 2002; Silva et al. 2002), the uterotrophic assay (Tinwell and Ashby 2004), and vitellogenin induction in fish (Brian et al. 2005). The basis of the something from nothing phenomenon derives from the theoretical assumptions that underlie the concept of dose addition. According to dose addition, every agent at any dose contributes, in proportion to its toxic unit, to the overall effect of a mixture. Because every mixture component can be replaced totally or in part by an equal fraction of an equi-effective dose of another, it does not matter whether the individual doses are also effective on their own. "Something from nothing" effects should occur even when individual toxicants are present at doses below effect thresholds, provided sufficiently large numbers of components sum up to a suitably high total-effect dose.

The results shown in Figure 3 support the idea that the "something from nothing" phenomenon also applies to alterations in the AGD of male rats exposed to anti-androgens during development. In this case a combination of 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone induced half-maximal AGD alterations, but the effects induced by each chemical on its own did not reach statistical significance when compared with effects in untreated controls. However, whether the doses of the chemicals present in the mixture were indeed equivalent to nothing in the sense of zero effect levels is debatable. Regression analysis of the dose–response data for the three chemicals (Figure 2) showed that the effects associated with these doses were between 5 and 10% of a biologically possible maximal effect. In addition, in the earlier dose–response study, vinclozolin actually induced a significant effect on AGD at a lower dose than the 24.5 mg/kg/day present in the mixture. Generally, these results show that lack of statistical significance cannot be equated with an absence of biological effects.

Because of the apparently greater sensitivity of NR as an anti-androgenic end point, the something from nothing effect could not be evaluated with a combination of 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone, because the individual doses induced NR that clearly reached statistical significance (Figure 3). The results, however, illustrate something not too dissimilar from the something from nothing phenomenon, which could be called "marked effects from small effects": The mixture-induced NR approaching complete feminization of the males, whereas the individual doses caused only modest effects. In general, our findings do not contradict theoretical expectations and are consistent with the earlier observations made with mixtures of estrogenic chemicals (Brian et al. 2005; Rajapakse et al. 2002; Silva et al. 2002; Tinwell and Ashby 2004). The something from nothing phenomenon would most probably have been demonstrated also with NR as the end point, had lower doses been employed or had more mixture components been combined.

In conclusion, our results show that combinations of similarly acting anti-androgens are able to produce developmental effects in male offspring of rats. These effects can be predicted fairly accurately on the basis of information about the potency of the individual mixture components by using the dose addition concept. There are indications that anti-androgens act together to produce marked joint effects when combined at doses that individually produce small, statistically insignificant responses. The significance of these findings for human and environmental risk assessment cannot be overstated; doses of endocrine-active chemicals, which appear to exert only small effects when judged on their own, may induce marked responses when they act in concert with numerous, possibly unrecognized, similarly acting agents.

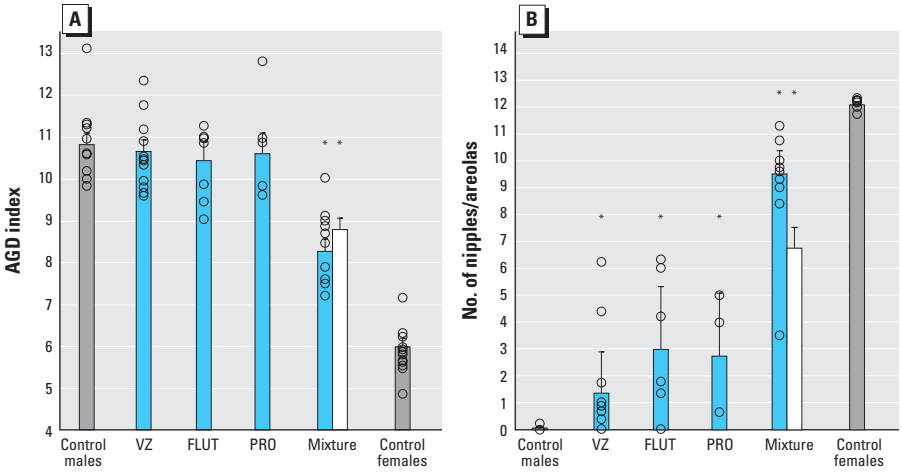

Figure 3. Mixture effects on AGD (*A*) and NR (*B*) at low doses of individual mixture components. Results shown are group mean ± 95% confidence belt for control males and females (gray), individual doses of 24.5 mg/kg vinclozolin (VZ), 0.77 mg/kg flutamide (FLUT), and 14.1 mg/kg prycymidone (PRO) (blue), the combined mixture dose of 39.37 mg/kg (blue), and the predicted mixture effect (white). Open circles represent litter means.
*$p < 0.05$ compared to control.

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Paper III

Metzdorff SB, Dalgaard M, Christiansen S, Axelstad M, Hass U, Kiersgaard MK, Scholze M, Kortenkamp A, Vinggaard AM. Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after in utero exposure to antiandrogen mixtures. Toxicological Sciences 98:87-98 (2007).

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Dysgenesis and Histological Changes of Genitals and Perturbations of Gene Expression in Male Rats after *In Utero* Exposure to Antiandrogen Mixtures

Stine Broeng Metzdorff,* Majken Dalgaard,* Sofie Christiansen,* Marta Axelstad,* Ulla Hass,*,[1]
Maria Kristina Kiersgaard,* Martin Scholze,† Andreas Kortenkamp,† and Anne Marie Vinggaard*

**Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark; and †The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, United Kingdom*

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We investigated the ability of a mixture of three androgen receptor antagonists to induce disruption of male sexual differentiation after perinatal exposure. The aim was to assess whether the joint effects of vinclozolin, flutamide, and procymidone can be predicted based on dose-response data of the individual chemicals. Chemicals were administered orally to pregnant Wistar rats from gestational day 7 to postnatal day 16. Changes in reproductive organ weights and of androgen-regulated gene expression in prostates from male rat pups were chosen as end points for extensive dose-response studies. With all end points, the joint effects of the three antiandrogens were dose additive. Histological evaluations showed that dysgenesis and hypoplasia of prostates, seminal vesicles, and epididymis were seen with the highest mixture doses. No changes were observed in any single-compound low-dose group for these lesions, nor were there histopathological changes in the testes. Pronounced dysgenesis of external genitals was observed with all doses of the mixture, and severe dysgenesis was seen with a mixture for which the individual compounds caused no effects. A combination of doses of each chemical that on its own did not produce significant reductions in the weights of seminal vesicles and *PBP C3* expression induced a marked mixture effect. Thus, antiandrogens cause additive effects on end points of various molecular complexities such as alterations at the morphological and the molecular level. Exposure to antiandrogens, which appears to exert only small effects when judged on a chemical-by-chemical basis, may induce marked responses in concert with, possibly unrecognized, similarly acting chemicals.

Key Words: **mixtures; androgen receptor antagonist; vinclozolin; flutamide; procymidone; developmental toxicity; gene expression; rat; endocrine disrupters.**

Since the early 1990's, an increasing incidence of disorders such as cryptorchidism and hypospadia in newborn boys, decreased sperm counts in young men, and a rising incidence of hormone-related testicular cancer have been observed in the human population. Each of these endocrine disorders can be associated with subnormal androgen action in fetal life which may lead to these reproductive abnormalities, also commonly characterized as the testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001). Environmental antiandrogens, which can arise from many different sources, including pesticides, industrial chemicals, pharmaceuticals, and phytochemicals, are potential endocrine disrupters. Such antiandrogens have the potential to perturb male reproductive development and act via a variety of mechanisms, including decreased androgen synthesis, disturbance of the pituitary-gonadal axis, and by blocking the androgen receptor (AR) (reviewed by Sharpe, 2006). Although evidence of a causal relationship between prenatal exposure and TDS is inherently difficult to establish in human studies, a few studies have recently found correlations between human levels of environmental antiandrogens (i.e., phthalates) and anogenital distance (AGD) (Swan *et al.*, 2005) and hormone levels in newborn boys (Main *et al.*, 2006), indicating that exposure to endocrine disrupters may cause developmental effects in human infants.

Human exposure to single antiandrogens is generally considered low. However, as several antiandrogenic chemicals have been found to occur as mixtures in humans (Blount *et al.*, 2000; Swan *et al.*, 2005), including children (Brock *et al.*, 2002; Main *et al.*, 2006) and in wildlife (Guillette, 2000), the consequences of combined exposures to antiandrogens warrant attention in order to assess the human health risk. Only a few studies have addressed mixture effects of endocrine disrupting chemicals, focusing on antiandrogenic effects *in vivo* (Birkhoj *et al.*, 2004; Gray *et al.*, 2001; Hass *et al.*, 2007; Nellemann *et al.*, 2003). Until recently, little was known about the developmental effects of *in utero* and early postnatal exposure to multiple antiandrogenic chemicals. Consequently, we designed a mixture study of three AR antagonists: vinclozolin and procymidone, two fungicides that share a common antiandrogenic mechanism, and flutamide, a pharmaceutical used to treat prostate cancer, to study disruption of male sexual differentiation in male rat pups after gestational and lactational exposure (Hass *et al.*, 2007).

[1] To whom correspondence should be addressed. Fax: +45-72347001. E-mail: ulh@food.dtu.dk.

Common developmental effects of all three single chemicals after perinatal exposure of male rats include altered AGD, nipple retention (NR), hypospadias, reduced reproductive organ weights, and altered behavior in male offspring (Foster and McIntyre, 2002; Gray *et al.*, 1994; Hellwig *et al.*, 2000; Hib and Ponzio, 1995; Hotchkiss *et al.*, 2002; McIntyre *et al.*, 2001; Miyata *et al.*, 2002; Ostby *et al.*, 1999; Shimamura *et al.*, 2002).

In a previous study (Hass *et al.*, 2007), we investigated whether the joint developmental effects of these three antiandrogens could be predicted based on dose-response data of the individual chemicals by employing the concept of dose addition (Loewe and Muischnek, 1926). In the paper by Hass *et al.* (2007), the focus was on AGD and NR as the end points for assessment, and the results revealed that the combined effects of the three antiandrogens were dose additive for AGD and that the observed responses for NR were slightly higher than those expected on the basis of dose addition. Doses of each chemical that individually did not induce statistically significant changes in AGD led to marked effects when combined as a mixture. Furthermore, as individual doses correlated with only modest effects on NR, the mixture induced NR that approached complete ''feminization'' of the males (Hass *et al.*, 2007). These results revealed that exposure to a mixture of antiandrogens with similar mechanism of action can induce marked effects even when each chemical is present at doses associated with only weak, if any, effects. This is consistent with theoretical expectations, and with earlier observations made with mixtures of estrogenic chemicals (Brian *et al.*, 2005; Rajapakse *et al.*, 2002; Silva *et al.*, 2002; Tinwell and Ashby, 2004), and it highlights the importance of these findings for human and environmental risk assessment.

In the present paper, we broaden the range of end points relevant to antiandrogen action and present additional results from our original three-component mixture study with vinclozolin, flutamide, and procymidone. Our interest was to explore whether combinations of these antiandrogens followed dose additivity when end points representative of antiandrogen action at different levels of biological complexity, ranging from the molecular to the macroscopic, were chosen as the basis for evaluation. The end points selected for quantitative dose-response analysis included reproductive organ weights and perturbations of gene expression in the prostate. An additional aim was to determine whether small effects induced by low doses of single-mixture components would lead to exacerbations when the chemicals acted in concert, as previously seen for AGD and NR. To fulfill this goal, malformations of male external genitals were scored in addition to histological lesions and weights of reproductive organs as well as prostate gene expression.

MATERIALS AND METHODS

Test compounds. Vinclozolin, 99% pure (CAS no. 50471-44-8) (Bie & Berntsen, ChemService cat. no. PS-1049), flutamide, 99% pure (CAS no. 13311-84-7) (Sigma Aldrich, Brøndby, Denmark cat. no. F9397), and procymidone, 99% pure (CAS no. 32809-16-8) (Bie & Berntsen, ChemService cat. no. PS-2126) were used. Test compounds were dissolved in corn oil (Bie & Berntsen, Herlev, Denmark), which was employed as vehicle.

Studies and dose levels. Dose-response studies for each test compound were performed prior to the mixture study in order to cover the entire range of effects, from no effect up to clear effects, without causing marked general toxicity to dams and offspring.

Dose-response study 1 involved a vehicle-dosed control group (16 dams), six doses of vinclozolin: 5, 10, 20, 40, 80, or 160 mg/kg/day and six doses of flutamide: 0.5, 1.0, 2.0, 4.0, 8.0, or 15 mg/kg/day (eight dams per dose group).

Dose-response study 2 included a vehicle-dosed control group (16 dams) and six doses of procymidone: 5, 10, 25, 50, 100, or 150 mg/kg/day (eight dams per dose group).

In the Mix study, a master mixture was prepared by combining doses of vinclozolin, flutamide, and procymidone that all induced a half-maximal degree of NR (six nipples) in male offspring. This approach was chosen in order to avoid that one single chemical contributed disproportionately to the overall mixture effect. The resulting mixture ratio of vinclozolin, flutamide, and procymidone was 0.62:0.02:0.36, and the master mixture contained 22.026 mg vinclozolin, 696.6 mg flutamide, and 12.675 mg procymidon in 600 ml corn oil. The master mixture was diluted into five dilutions termed 'Mix1' to 'Mix5'. These five solutions gave rise to the following total doses of all three chemicals combined: 7.9, 19.7, 39.3, 70.8, or 106.2 mg/kg/day (16 dams per dose group). The Mix study also included a vehicle-dosed control group (16 dams) and a low and high dose of the three single compounds: vinclozolin (24.5 and 95.9 mg/kg/day), flutamide (0.77 and 3.9 mg/kg/day), and procymidone (14.1 and 68.1 mg/kg/day). For vinclozolin, 16 dams per dose group and 8 dams per dose group for the latter two compounds were included. It should be noted that the low doses of all three chemicals were combined to give one of the doses in the Mix study ('Mix3', 39.3 mg/kg/day).

Animals and dosing. Time-mated young adult nulliparous Wistar rats (HanTac:WH, Taconic Europe, Denmark, body weight approximately 200g) were supplied at day 3 of pregnancy (gestational day [GD] 3). The day after arrival, at GD 4, the dams were randomly distributed into groups of 8 or 16 animals with similar body weight distribution. The animals were housed and handled as previously described (Hass *et al.*, 2007). Test compounds and mixtures were administered by gavage from GD 7 to the day prior to expected birth (GD 21) and from postnatal day (PND) 1 to PND 16. Dams were weighed daily, and the health status of dams and offspring was monitored twice daily. All studies were divided into four blocks (one week between blocks), and each dose group was represented equally in all four blocks.

Autopsy of male pups PND 16. The body weights of male pups were recorded, and an autopsy was performed at PND 16. Male external genitals were investigated, various organs were excised and weighed, and used for either histopathology or gene expression studies. Trunk blood was taken and pooled within litters.

Investigation of external genitals. At PND 16, the external genitals were inspected blinded to the observer in all males from all litters. The changes were scored on a scale from 0 to 3 in order to investigate if male external genitals were demasculinized. The scores were as follows:

Score 0 (no effect): normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact. In the perineal area, thick fur extends caudally from the base of the genital tubercle and half the distance to the anus. A furless area circumscribes the anus.

Score 1 (mild dysgenesis of the external genitals): a small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening is observed, estimated 0.5–1.4 on an arbitrary scale. The size of the genital tubercle may be decreased. The furless area around the anus expands toward the base of the genital tubercle, but thick fur is still present at the base of the genital tubercle.

Score 2 (moderate dysgenesis of the external genitals): the preputial cleft is larger, estimated 1.5–2.4 on an arbitrary scale. The urethral opening is situated halfway down toward the base of the genital tubercle (hypospadia). Partly furless e.g., thin fur is noted in the perineal area ranging from the base of the genital tubercle and caudally to the furless area circumscribing the anus.

Score 3 (severe dysgenesis of the external genitals): the preputial cleft is large, estimated 2.5–3.5 on an arbitrary scale. The urethral opening is situated further than halfway down the inferior side of the genital tubercle to the base of the genital tubercle. At the base of the genital tubercle, a groove extending laterally is observed (similar to control females at PND 16). The rat is totally furless in the entire perineal area.

Dissection and histopathology of organs. From one male per litter at PND 16, the following organs were excised and weighed: testis, epididymides, ventral prostate, seminal vesicles, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands, adrenals, kidney, and liver. From one or two males per litter, right or left testis was alternately fixed in Bouin's fixative. From males in the Mix study, the thyroid glands were excised and weighed as well. The remaining aforementioned organs, and one testis from litters with three or more males, were fixed in formalin. All fixed organs were embedded in paraffin, stained with hematoxylin and eosin, and used for the histopathological evaluation.

Gene expression levels. When at least two males were in a litter, one pub was randomly selected and its ventral prostate was weighed and stored in RNA*later* (Qiagen, Ballerup, Denmark) for gene expression analyses. The organs were homogenized, and total RNA was isolated using RNeasy-mini kit and RNase-Free DNase set (Qiagen). cDNA was synthesized from 0.5 µg total RNA using the Omniscript Reverse Transcription kit (Qiagen) with T16 oligonucleotides and a 18S rRNA primer. Samples were quantified on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Naerum, Denmark) by standard TaqMan technology.

Expression levels of *prostate-binding protein subunit C3* (*PBP C3*), *ornithine decarboxylase* (*ODC*), *insulin-like growth factor I* (*IGF-I*), *complement component 3* (*Compl.C3*), *testosterone-repressed prostate message 2* (*TRPM-2*), and *AR* were quantified in the prostate. Expression levels of each target gene were normalized to the expression level of the housekeeping gene *18S rRNA*. For each sample, 2-µl cDNA (1.75 ng/µl) was amplified under universal thermal cycling parameters (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 10 µl. Three separate amplifications were performed for each gene, and when intraassay variation was above 15%, additional amplifications were performed. All genes were quantified from standard curves. Primers and probes for *PBP C3*, *ODC*, *IGF-1*, *Compl.C3*, *TRPM-2*, *AR*, and *18S rRNA* have previously been published by Laier *et al.* (2006). The number of prostates analyzed from the control and the Mix1 to Mix5 group was 12, 11, 12, 11, 10, and 4, respectively. The number of prostrates from the low and high dose groups of single chemicals (Vin, Flu, and Pro) were 12, 10, 6, 4, 4, and 6, respectively. All prostates were from males belonging to different litters.

Statistical data analyses. The weights of male and female pups were analyzed for all offspring. Statistical analyses of organ weights and macroscopic lesions were performed for one to four males per litter, and testes were analyzed from all offspring. The numbers of litters varied for the control and mixture groups from 11 to 13 and for the single-compound experiments from 4 to 10, respectively.

Continuous data were confirmed for normal distribution and homogeneity (Shapiro-Wilk's and Bartlett's tests). Dose-response effects different from controls were estimated by multiple testing methods (global error rate $\alpha = 5\%$, two sided). When organ weights were analyzed, body weight was included as covariate, and statistical analyses were always adjusted using litter as an independent, random, and nested factor. Statistical significance was then assessed on the basis of contrast tests. All analyses were done using the SAS procedure PROC GENMOD, PROC MIXED, and PROC MULTTEST (SAS version 8, SAS Institute Inc., Cary, NC). Macroscopic and histological lesions were analyzed using Fisher's exact test with p values adjusted by permutation.

Statistical dose-response regression analyses for body weight data (seminal vesicle, ventral prostate, and LABC) and gene expression data (PBP C3/18S) were carried out by applying a best-fit approach (Scholze *et al.* 2001). Various nonlinear regression models (logit, probit Weibull, generalized logit) were fitted independently to the same data set, and the best fitting model was selected on the basis of a statistical goodness-of-fit criterion (information criterion of Schwarz). To control for litter effects, dose-response data were analyzed by using a generalized nonlinear mixed modeling approach (Vonesh and Chinchilli, 1996), with litter as a random effect modifier for individual organ weights and carried out using the SAS procedure PROC NLMIXED.

Prior to regression analysis, we analyzed whether there was a linear relationship between organ weights and body weight based on all control pups. This was indeed the case, and the corresponding regression lines went through the origin within their 95% confidence belts. Therefore, individual organ weights were normalized as the ratio between organ and body weight. Regression analyses were done for the single compounds on pooled effect data from the initial dose-response studies (Table 1) and the repeated doses that were run concurrently with the mixture study (Table 2).

Under assumption of additive combination effects, a dose-response relationship for the mixture was predicted using the best-fit dose-response regression curves of the individual compounds and compared to the observed effects. Equation 1 allows calculation of any effect dose of a mixture under the hypothesis of dose additivity, provided the dose-response functions of all mixture components and the mixture ratio are known

$$\mathrm{EDx}_{mixture} = \left(\frac{p_1}{\mathrm{EDx}_1} + \frac{p_2}{\mathrm{EDx}_2} + \frac{p_3}{\mathrm{EDx}_3} \right)^{-1} \quad (1)$$

EDx_1, EDx_2, and EDx_3 are the effect doses of vinclozolin, flutamide, and procymidone that on their own produce the same quantitative effect x as the mixture, and p_1, p_2, and p_3 are the relative proportions of the corresponding individual doses present in the total mixture dose. The statistical uncertainty for the predicted mixture effects was determined by using the bootstrap method (Efron and Tibshirani, 1993) and expressed as 95% confidence limits for the predicted mean estimate.

RESULTS

Pregnancy Data, Postnatal Growth, and General Toxicity

No clinical signs of general toxicity were observed. The maternal body weight gain from GD 7 to PND 1, pregnancy length, litter size, birth weight of male and female offspring, sex ratio in the litters, and pup weight gain and survival were unaltered in all groups when compared to controls.

Malformations of the External Male Genitals

In the Mix study, external male genitals were investigated for malformations. For all low mixture doses (Mix1–Mix3), between 30 and 70% of the pups were diagnosed with a mild dysgenesis (score 1). At the higher doses, moderate (score 2) and severe dysgenesis (score 3) dominated, with up to 100% of the animals affected in the experiments with the two highest doses (Fig. 1A). Overall, the incidence as well as the severity of malformations increased with increasing mixture doses. For all three single compounds, pups with a mild dysgenesis were observed only at the low doses, while animals with a moderate

TABLE 1

Dose-Response Studies of Vinclozolin, Procymidone, and Flutamide in Which Pregnant Rats Were Exposed from GD 7 to PND 16. Body and Organ Weights from Male Rat Pups PND 16 are Shown

	Body weight (g)	Testis right (mg)	Testis left (mg)	Epididymides (mg)	Ventral prostate (mg)	Seminal vesicle (mg)	LABC (mg)	Bulbourethral glands (mg)	Adrenals (mg)	Kidneys (mg)	Liver (mg)
Vinclozolin (mg/kg/day)											
Control	34.7 ± 1.1	66.0 ± 1.5	66.4 ± 1.2	27.0 ± 1.1	16.5 ± 0.6	12.0 ± 0.9	27.0 ± 1.8	2.2 ± 0.2	10.5 ± 0.3	347 ± 14.1	895 ± 31.2
5	33.5 ± 1.2	61.1 ± 1.1	62.0 ± 1.0*	25.5 ± 3.9	13.7 ± 0.7	9.2 ± 0.1	24.0 ± 2.6	1.9 ± 0.2	10.4 ± 0.6	328 ± 19.2	855 ± 35.3
10	34.3 ± 0.8	64.7 ± 1.1	65.5 ± 1.2	**21.2 ± 1.8***	**15.1 ± 0.5***	**10.0 ± 0.5***	27.4 ± 1.7	2.0 ± 0.2	11.3 ± 0.5	340 ± 15.4	873 ± 34.8
20	34.8 ± 1.3	68.0 ± 1.5	67.7 ± 1.6	**21.8 ± 0.5****	**14.8 ± 0.7***	**10.3 ± 0.6***	25.0 ± 0.5	1.7 ± 0.2	10.7 ± 0.5	360 ± 18.6	906 ± 43.4
40	34.3 ± 1.1	68.5 ± 1.2	68.7 ± 1.2	**22.2 ± 1.6****	15.0 ± 0.6	**9.7 ± 1.0***	22.7 ± 2.0	1.5 ± 0.2	10.8 ± 0.2	344 ± 14.4	876 ±4 0.9
80	34.9 ± 0.9	66.7 ± 1.7	67.9 ± 1.7	**22.3 ± 1.8****	**10.5 ± 0.6****	**7.3 ± 0.7****	**20.4 ± 2.2***	**1.1 ± 0.3***	**12.8 ± 0.8****	357 ± 14.8	961 ± 29.2
160	34.1 ± 1.6	**55.2 ± 2.7***	**54.9 ± 2.6****	**17.1 ± 1.0****	**5.1 ± 1.0****	**3.1 ± 0.8****	**12.1 ± 1.6****	**ND****	**12.5 ± 0.5****	334 ± 24.1	**920 ± 71.2***
Flutamide (mg/kg/day)											
Control	34.7 ± 1.1	66.0 ± 1.5	66.4 ± 1.2	27.0 ± 1.1	16.5 ± 0.6	12.0 ± 0.9	27.0 ± 1.8	2.2 ± 0.2	10.5 ± 0.3	348 ± 14.1	895 ± 31.2
0.5	34.1 ± 1.3	66.3 ± 1.5	65.7 ± 1.5	23.9 ± 0.7	**13.8 ± 0.8***	**9.9 ± 0.3***	24.9 ± 2.0	1.7 ± 0.2	9.5 ± 0.7	335 ± 16.9	895 ± 39.9
1	32.7 ± 0.6	61.8 ± 1.1	62.6 ± 1.2	**22.7 ± 1.5***	**13.2 ± 0.6****	11.0 ± 0.7	24.8 ± 1.9	2.2 ± 0.6	11.1 ± 0.3	326 ± 11.9	843 ± 36.0
2	34.6 ± 1.1	67.4 ± 1.6	67.8 ± 1.7	26.7 ± 1.6	**10.9 ± 0.7****	**9.3 ± 0.6***	22.6 ± 1.1	1.6 ± 0.1	9.9 ± 0.5	345 ± 9.8	884 ± 34.3
4	36.4 ± 0.6	69.4 ± 1.4	68.2 ± 1.4	24.1 ± 1.8	**8.6 ± 0.5****	**6.8 ± 1.2****	20.5 ± 2.5	1.5 ± 0.4	9.9 ± 0.5	375 ± 14.9	948 ± 42.1
8	35.6 ± 1.3	64.8 ± 1.3	64.6 ± 1.3	**20.5 ± 1.2****	**3.3 ± 0.4****	**3.8 ± 1.0****	**14.9 ± 3.6***	1.5 ± 0.1	11.9 ± 0.9	354 ± 19.6	935 ± 42.7
16	34.8 ± 1.1	**56.0 ± 2.0****	**56.1 ± 1.6****	**17.4 ± 0.3****	**2.3 ± 0.4****	**2.0 ± 0.2****	**11.1 ± 2.8****	**ND****	10.5 ± 0.9	352 ± 15.1	914 ± 28.1
Procymidone (mg/kg/day)											
Control	38.5 ± 1.2	68.7 ± 1.7	68.9 ± 1.8	25.3 ± 1.2	16.6 ± 0.8	13.4 ± 1.2	24.8 ± 1.4	1.9 ± 0.1	10.9 ± 0.4	399 ± 14.3	1032 ± 27.9
5	39.3 ± 1.5	69.5 ± 1.2	69.7 ± 1.2	25.9 ± 1.5	14.7 ± 0.8	**10.2 ± 0.8***	22.1 ± 2.1	1.7 ± 0.1	10.8 ± 0.7	405 ± 17.6	1031 ± 54.5
10	36.1 ± 1.4	**70.0 ± 1.3***	**69.8 ± 1.2***	24.6 ± 1.6	**13.0 ± 0.6***	11.9 ± 0.9	**20.3 ± 1.3***	**1.5 ± 0.0***	9.9 ± 0.7	383 ± 23.3	923 ± 40.4
25	35.9 ± 2.5	67.0 ± 2.9	67.0 ± 3.5	**20.0 ± 1.3***	**10.1 ± 1.0****	**8.5 ± 1.0****	23.0 ± 3.8	**1.3 ± 0.2****	11.1 ± 0.8	373 ± 31.5	962 ± 83.0
50	36.8 ± 1.9	68.1 ± 2.0	68.6 ± 1.9	22.0 ± 1.6	**9.1 ± 0.8****	**8.4 ± 0.9****	**17.4 ± 1.1****	**0.9 ± 0.2****	10.9 ± 0.6	385 ± 22.8	994 ± 55.3
100	32.0 ± 1.8	61.0 ± 1.5	60.7 ± 1.6	**18.4 ± 2.0****	**6.2 ± 1.0****	**6.6 ± 1.1****	19.7 ± 2.0	**0.7 ± 0.5****	10.8 ± 0.7	333 ± 9.5	916 ± 32.6
150	36.0 ± 1.5	**54.0 ± 1.6****	**54.1 ± 1.8****	**16.8 ± 1.6****	**2.3 ± 0.4****	**4.5 ± 0.9****	**9.4 ± 1.9****	**1.2 ± 0.4***	**13.0 ± 1.2***	378 ± 22.0	1123 ± 111.8

Note. Data represent least squares means ± SEM. ND, not detectable, tissue weights were too low to measure.

The columns 2, 3, 4, 5, 9, 10, and 11 of ''Vinclozolin (mg/kg/day)'' and ''Flutamide (mg/kg/day)'' and the columns 2, 3, 5, 9, and 10 of ''Procymidone (mg/kg/day)'' represent statistical significant effect of the covariate, body weight ($p < 0.05$).

Statistical significant different compared to controls (*$p < 0.05$) and (**$p < 0.01$), respectively. All statistically significant numbers are shown in bold.

TABLE 2

Mixture Study: Five Mixtures of Vinclozolin (Vin), Procymidone (Pro), and Flutamide (Flu) Were Administered to Pregnant Rats from GD 7 to PND 16. Body and Organ Weights from Male Rat Pups PND 16 are Shown. In Addition a Low and a High Dose of Each Single Compound Were Included in the Experiment

Group (dose in mg/kg/day)	Body weight (g)	Right testis (mg)[a]	Left testis (mg)[a]	Epididymides (mg)[a]	Ventral prostate (mg)	Seminal vesicle (mg)[a]	LABC (mg)[a]	Bulbourethral glands (mg)[a]	Adrenals (mg)[a]	Kidneys (mg)[a]	Liver (mg)[a]	Thyroid gland (mg)[a]
Control	33.7 ± 0.9	64.4 ± 2.2	65.1 ± 2.3	25.3 ± 0.8	17.1 ± 0.9	11.0 ± 0.9	31.1 ± 1.8	2.5 ± 0.3	10.1 ± 0.3	350 ± 8.6	872 ± 29.1	4.2 ± 0.3
Mix1 (7.9)	33.7 ± 1.1	62.7 ± 2.0	63.8 ± 1.8	**22.1 ± 0.8****	15.4 ± 1.4	10.2 ± 0.9	29.6 ± 1.8	1.9 ± 0.2	10.5 ± 0.5	349 ± 14.1	847 ± 30.2	4.0 ± 0.2
Mix2 (19.7)	33.9 ± 1.4	65.9 ± 1.9	66.8 ± 1.9	**21.9 ± 0.7****	**12.4 ± 0.5****	8.5 ± 0.7	26.7 ± 1.4	**1.4 ± 0.1***	10.5 ± 0.4	357 ± 13.9	860 ± 48.5	3.6 ± 0.2
Mix3 (39.3)	32.2 ± 1.3	62.7 ± 3.2	63.7 ± 3.4	**18.8 ± 0.9****	**9.4 ± 0.6****	**6.4 ± 0.7***	**17.6 ± 1.9***	**0.7 ± 0.1****	10.5 ± 0.7	330 ± 15.2	830 ± 42.8	4.0 ± 0.1
Mix4 (70.8)	30.6 ± 1.1	**55.6 ± 1.8***	57.0 ± 1.9	**15.9 ± 0.9****	**4.8 ± 0.8****	**3.8 ± 0.8****	**13.7 ± 0.6****	**1.2 ± ·****	**11.2 ± 0.3***	322 ± 16.9	**843 ± 44.0***	3.6 ± 3.2
Mix5 (106.2)	32.0 ± 1.7	**50.4 ± 2.7***	**51.6 ± 2.4***	**11.6 ± 0.8****	**2.0 ± 0.6****	**2.1 ± 0.5****	**8.2 ± 0.9****	**0.3 ± ·****	**11.5 ± 0.7****	321 ± 22.8	**845 ± 52.5***	3.4 ± 0.2
Vin (24.5)	34.1 ± 1.9	64.7 ± 3.0	65.4 ± 3.1	**21.8 ± 0.7****	**13.8 ± 0.6****	9.4 ± 0.7	**25.1 ± 1.4***	**1.6 ± 0.2***	10.7 ± 0.6	351 ± 19.2	867 ± 57.3	**3.5 ± 0.2***
Vin (95.9)	32.7 ± 1.2	60.1 ± 2.1	61.0 ± 2.1	**17.2 ± 0.7****	**7.9 ± 0.6****	**4.9 ± 0.8****	**18.7 ± 1.7****	**0.6 ± 0.2****	**11.5 ± 0.4***	343 ± 16.5	**855 ± 38.7***	**3.8 ± 0.2***
Flu (0.77)	36.4 ± 2.0	68.3 ± 3.2	68.4 ± 3.4	**22.4 ± 1.1***	15.5 ± 0.5	8.3 ± 1.0	27.2 ± 2.0	2.0 ± 0.3	10.3 ± 0.7	376 ± 26.6	920 ± 58.3	4.5 ± 0.7
Flu (3.86)	37.5 ± 3.7	70.3 ± 7.3	69.6 ± 7.2	**20.8 ± 2.7***	**11.9 ± 1.3****	**6.6 ± 1.5***	**21.4 ± 2.1***	1.0 ± ·	10.7 ± 1.4	416 ± 55.5	1020 ± 123.6	4.5 ± 0.6
Pro (14.1)	37.1 ± 1.8	72.7 ± 3.7	72.2 ± 3.1	**21.8 ± 1.0***	**14.8 ± 1.4***	8.9 ± 0.9	28.1 ± 0.8	1.8 ± 0.2	10.6 ± 0.4	357 ± 22.9	959 ± 30.1	4.6 ± 0.5
Pro (68.1)	31.6 ± 1.5	57.7 ± 4.2	58.6 ± 4.2	**16.1 ± 1.3****	**8.8 ± 0.6***	**5.9 ± 1.4***	**16.8 ± 1.2****	0.1 ± ·	10.5 ± 0.4	338 ± 23.7	**884 ± 38.5***	3.6 ± 0.4

Note. Data represent least squares means ± SEM. '·' Denotes that SEM is close to 0.

[a]Statistical significant effect of the covariate, body weight ($p < 0.05$).

Statistical significant different compared to controls (*$p < 0.05$) and (**$p < 0.01$), respectively. All statistically significant numbers are shown in bold.

FIG. 1. (A) Frequency of dysgenesis of external genitals in male offspring after exposure to either vehicle, five doses of the mixture, or low (L) and high (H) doses of vinclozolin (Vin), flutamide (Flu), and procymidone (Pro). Percent affected male offspring with mild (score 1), moderate (score 2), or severe (score 3) dysgenesis is shown. The number of rats investigated in the control and the Mix1–5 groups were 36, 48, 47, 39, 49, and 20, respectively, and were in the low and high dose groups of Vin, Flu, and Pro 24, 15, 39, 36, 14, and 29, respectively. (B) The incidence of unilateral or bilateral epididymal dysgenesis is shown. The number of rats investigated in the control and the Mix1–5 groups were 36, 48, 47, 39, 49, and 20, respectively, and were in the low and high dose groups of Vin, Flu, and Pro 24, 15, 39, 36, 14, and 29, respectively. Data were analyzed by a Fisher's exact test. *Statistically significant effects compared to controls ($p < 0.05$).

(score 2) or severe (score 3) dysgenesis were diagnosed at the high doses.

Concerning the number of affected males with macroscopic epididymal dysgenesis (uni- or bilateral), a clear dose-dependent effect was identifiable for the mixture (Fig. 1B).

Mix3, a combination of 24.5 mg/kg vinclozolin, 0.77 mg/kg flutamide, and 14.1 mg/kg procymidone, yielded a clear increase in malformations. This response was considerably higher than any effect observed with the individual chemicals at the doses present in the combination.

Organ Weights

In the dose-response studies with the individual compounds, all reproductive organ weights showed a downward trend with increasing doses (Table 1). For vinclozolin, prostate, seminal vesicle, and epididymides weights were significantly reduced at 10 mg/kg and the remaining investigated organs, except kidneys, only at the higher doses (80 and 160 mg/kg). For prostate and seminal vesicle weights, these differences can be explained in terms of lower data variations with consequent lower statistical detection limits. A similar pattern became apparent for flutamide, although for all administered doses, the weights of the bulbourethral glands, adrenals, kidneys, and livers could not be detected as statistically different from the controls. For procymidone, seminal vesicle weights were affected at the lowest dose (5 mg/kg), weights of prostate, LABC, and bulbourethral glands were affected at 10 mg/kg, and epididymides weights were reduced at 25 mg/kg. Changes in the weight of the kidney and liver could not be identified.

In the Mix study, increasing doses both for the mixtures as well as for the single compounds decreased the reproductive organ weights (Table 2). A mixture dose of 19.7 mg/kg (Mix2) reduced weights of epididymides, prostates, and bulbourethral glands. The weight of the epididymides was the most sensitive parameter among all analyzed reproductive organs. At the Mix3, dose weights of all reproductive organs except for testes were reduced. In general, all high doses of single compounds affected reproductive organ weights, whereas low doses only affected prostate and LABC weights in a few cases (Table 2).

Histopathological Effects

Histopathological effects in the ventral prostate, seminal vesicles, epididymides, and in the testes were investigated in the Mix study. The ventral prostates from 16-day-old male rats are shown in the upper three Figures (2A for vehicle controls, 2B for mixture dose Mix4, 2C for mixture dose Mix5). Compared to controls (Fig. 2A), the three highest mixture doses and all high doses of the single compounds induced a dose-dependent hypoplasia of the ventral prostate, with a severe dysgenesis at the two highest mixture doses (Mix4 and Mix5). The acini were small, and the tall cuboidal epithelial cells lining the acini were flattened in some areas (Fig. 2B). The interstititial tissue was enlarged, and the acini were devoid of secretion (Fig. 2C).

A general hypoplasia was also seen in the seminal vesicle at the two highest mixture doses (Figs. 2E and 2F). In contrast to controls (Fig. 2D), exposed males with hypoplastic seminal vesicles lacked large ducts with papillary and villous projections (Fig. 2E). The epithelial cells lining the duct were flattened, sometimes more cuboidal than columnar, sometimes even almost squamous. In Mix5, the duct was primitively developed and was surrounded by extensive interstitial connective tissue (Fig. 2F). In the epididymides, the epithelial cells lining the tubule were occasionally small with pyknotic nuclei. Furthermore, the cells were disarranged and the tubule had no lumen (data not shown).

The incidence of male pups with histological alterations in the prostates, seminal vesicles, and epididymides are summarized in Figure 3, demonstrating that these three organs were all affected at higher mixture doses (Mix4, Mix5). For epididymides, data indicate a low incidence even at the lowest administered mixture doses but no effect of the single compounds. No changes were observed in any of the single-compound low-dose groups for any of the three organs, and there were no histopathological alterations in the testes in any of the groups in the Mix study (data not shown).

Gene Expression

The mRNA levels of the five androgen-regulated genes *PBP C3*, *ODC*, *IGF-1*, *Compl.C3*, and *TRPM-2* as well as those for the *AR* were investigated in ventral prostates by real-time RT-PCR in the two dose-response studies. With the aim of screening for altered gene expression to select biomarkers for the Mix study, only controls and the highest doses were examined. The experiments revealed that *PBP C3* and *ODC* mRNA were significantly down-regulated for vinclozolin and procymidone, and a tendency toward down-regulation was seen for flutamide as well. Furthermore, *Compl.C3* mRNA was significantly elevated in the prostates of rats that received flutamide and procymidone (data not shown). In the Mix study, all five androgen-regulated genes were investigated in the ventral prostate for all dose levels (Fig. 4). As expected, *PBP C3*, *ODC*, and *Compl.C3* mRNA were markedly affected in the mixture experiment. A significant down-regulation of *PBP C3* mRNA was found for Mix2 doses and higher, a down-regulation of *ODC* mRNA was evident at the Mix4 dose and higher, and finally an up-regulation of *Compl.C3* from the Mix2 dose. For the single compounds, *PBP C3* and *Compl.C3* were affected with the highest vinclozolin dose, *PBP C3* was down-regulated by procymidone, whereas flutamide showed no effects.

Observed versus Predicted Effect of Organ Weights and PBP C3 Expression

Dose-response relationships for organ weights (LABC, seminal vesicles, and prostate) and *PBP C3* expression in prostates were constructed for each of the three compounds, vinclozolin, flutamide, and procymidone (Fig. 5, left column). These data enabled us to calculate response curves for the mixture of the three compounds, under the assumption of dose addition. The anticipated (additive) responses were then compared with the experimentally observed mixture effects

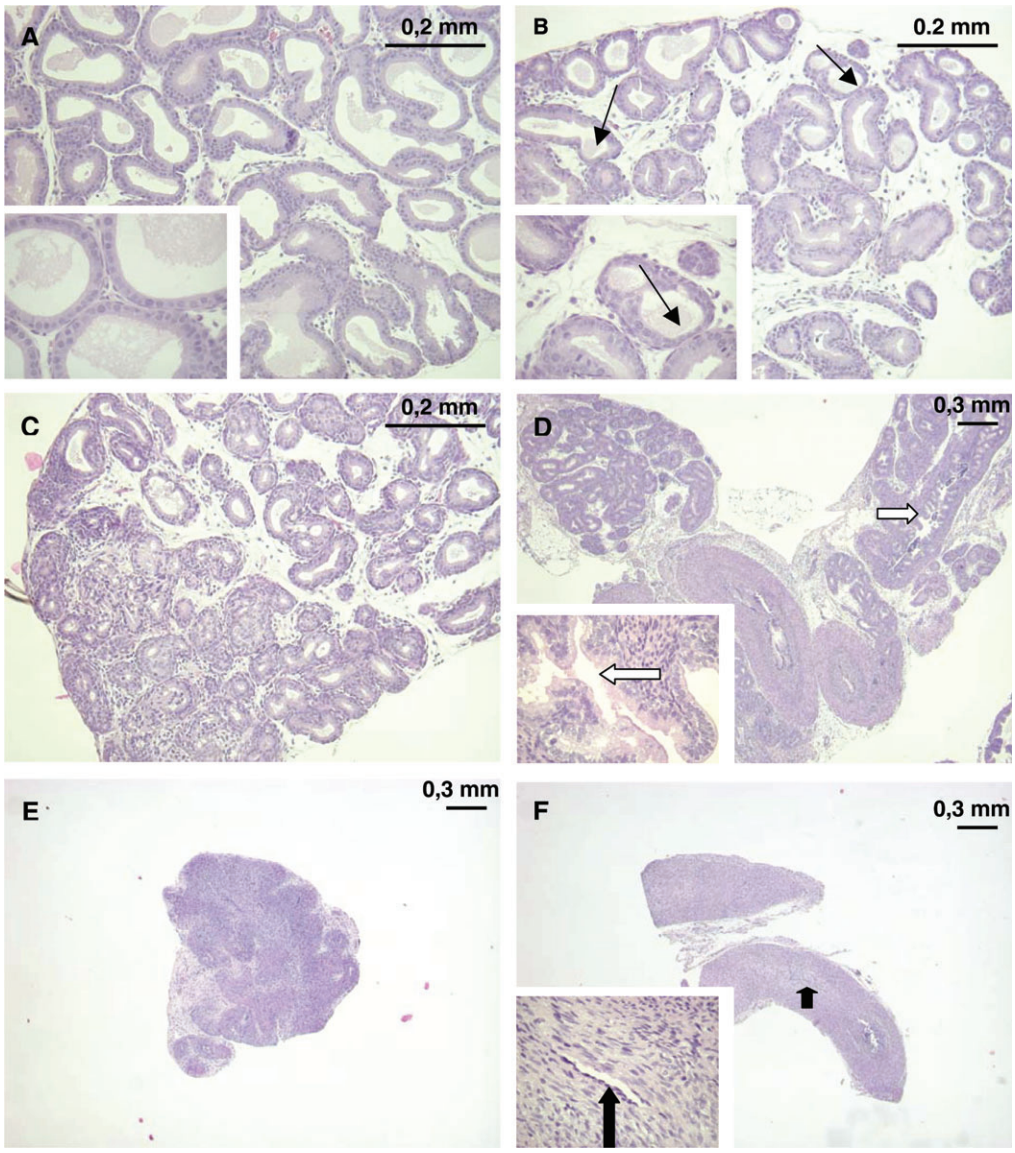

FIG. 2. Hematoxylin staining of reproductive organs from PND 16 male rats exposed to a mixture of the three AR antagonists vinclozolin, flutamide, and procymidone. Ventral prostates are shown from pups of the vehicle controls (A), Mix 4 group (70.8 mg/kg) (B), and Mix 5 group (106.2 mg/kg) (C). Seminal vesicles are shown from pups of the vehicle controls (D), Mix 4 group (E), and Mix 5 group (F). (A) In prostate from controls, large cubic to columnar epithelial cells with large amounts of cytoplasm line the acini of various sizes. Large, distended acini lined with tall cubic epithelial cells are arranged in the center of the gland, while the smaller and less distended acini lined with columnar epithelial cells are situated in the periphery. The acini are regular in shape and almost all acini contain secretion. The stroma is relatively scarce. (B) In general, the gland is hypoplastic, and the acini are small. In several areas, the epithelial cells are flattened (thin arrow). (C) In general, the gland is hypoplastic. The acini are quite small and irregular and only a few acini contain secretion. The epithelial cells are hypotrophic. The interstitial stroma is extended. (D) Seminal vesicles of a PND 16 control male rat. Note the papillary and villous infolding of the columnar epithelium (thick white arrow). (E) Seminal vesicles from a PND 16 male rat in Mix 4 group. The gland is hypoplastic. No papillary and villous infolding is seen. Epithelial cells are more cubic than columnar. A large amount of stroma is present compared to the seminal vesicles in control males. (F) Seminal vesicles from a PND 16 male rat in the Mix 5 group. The gland is hypoplastic, and only a primitive duct is visible. The epithelium lining the duct is exceedingly flattened (thick black arrow). A large amount of interstitial stroma cells is present.

(Fig. 5, right column). For all end points, the means of the observed mixture effects fell within the 95% confidence interval of the prediction curves, thus demonstrating good agreement with the expected additive effects of the compounds. It is safe to conclude that the overall combination effects were dose additive in all cases.

For each of the three AR antagonists and their mixture, the investigated end points showed comparable sensitivity. Thus, flutamide was most effective in the dose range between 1 and 10 mg/kg and procymidone and vinclozolin between 10 and 100 mg/kg, no matter whether reproductive organ weights or *PBP C3* expression was considered. Similarly, the investigated

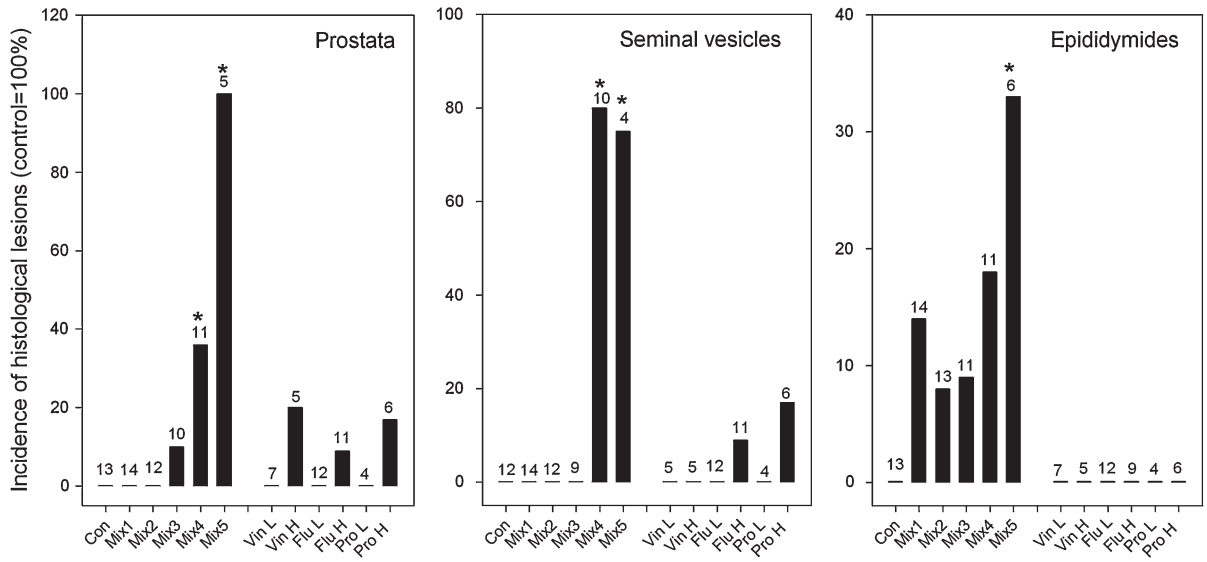

FIG. 3. The percentage incidence of histological alterations in ventral prostates, seminal vesicles, or epididymides in male rats pups (PND 16) is shown. The male pups were exposed to either vehicle, five doses of the mixture of vinclozolin (Vin), flutamide (Flu), and procymidone (Pro), or to low (L) or high (H) doses of the individual compounds. The numbers above the bars indicate the number of animals investigated in each group. The control group was set to 100%. *Statistically significant effects compared to controls ($p < 0.05$).

mixture was active between 10 and 100 mg/kg for all end points. Our previously published data show that AGD and NR exhibited comparable sensitivity to these three chemicals (Hass *et al*. 2007). Thus, all five end points of antiandrogen action, representative of differing levels of biological complexity (from the molecular to the organ level), appear to be equally well suited for assessments of mixture effects.

Finally, we tested whether doses of vinclozolin, flutamide, and procymidone that on their own were not effective or showed only small effects could induce significant responses in combination (Fig. 6). Administered on their own, vinclozolin (24.5 mg/kg), flutamide (0.77 mg/kg), and procymidone (14.1 mg/kg) did generally not induce effects statistically significantly different from vehicle-treated controls, the only exceptions being vinclozolin with respect to LABC weights (Fig. 6a) and ventral prostate weights and procymidone relative to ventral prostate weights (Fig. 6b). However, in all cases, the observed mixture effects were severe and statistically significant. Moreover, they were always higher than observed for the low doses of the individual compounds and were reasonably well predictable by dose addition.

DISCUSSION

Early work with antiandrogens focused mainly on events surrounding AR binding and activation and has shown that combinations of these chemicals are able to act together in an additive fashion (Birkhoj *et al.*, 2004; Nellemann *et al.*, 2003). These early studies were expanded upon by a recent paper addressing the question as to whether there are also joint effects with responses further removed from AR binding and activation, such as those related to male sexual differentiation (Hass *et al.*, 2007). In this study, a fixed mixture ratio approach was applied for assessing combination effects on the disruption of male sexual development as determined by alterations of AGD and NR in newborn rat males. Using these morphological end points as a sign of demasculinization of the males, we have shown that vinclozolin, flutamide, and procymidone exhibited dose additivity (Hass *et al.*, 2007). By making use of material from this study, we have investigated a wider range of end points indicative of antiandrogen action in 16-day-old male pups. The selected end points were morphological changes as determined by reproductive organ weights, dysgenesis of male external genitals, histological changes in the reproductive system, and changes at the molecular level as determined by gene expression levels in the prostate. It was our hypothesis that the joint action of vinclozolin, flutamide, and procymidone should also be dose additive in relation to these end points.

The results in our study show clearly that antiandrogens with a similar mode of action (AR antagonism) work together in an additive way for a broad spectrum of end points, ranging from reproductive organ weights to *PBP C3* gene expression in the prostate. Our findings are consistent with the previously reported additivity of antiandrogens on other end points such as AR receptor activation *in vitro* and *in vivo* (Birkhoj *et al.*, 2004; Nellemann *et al.*, 2003) and demasculinization of newborn males exposed during gestation and lactation (Hass *et al.*, 2007). Thus, the accumulated evidence points to the fact that receptor-mediated antiandrogenic effects follow the dose-addition principle for various end points of differing biological complexity, ranging from changes at the morphological level,

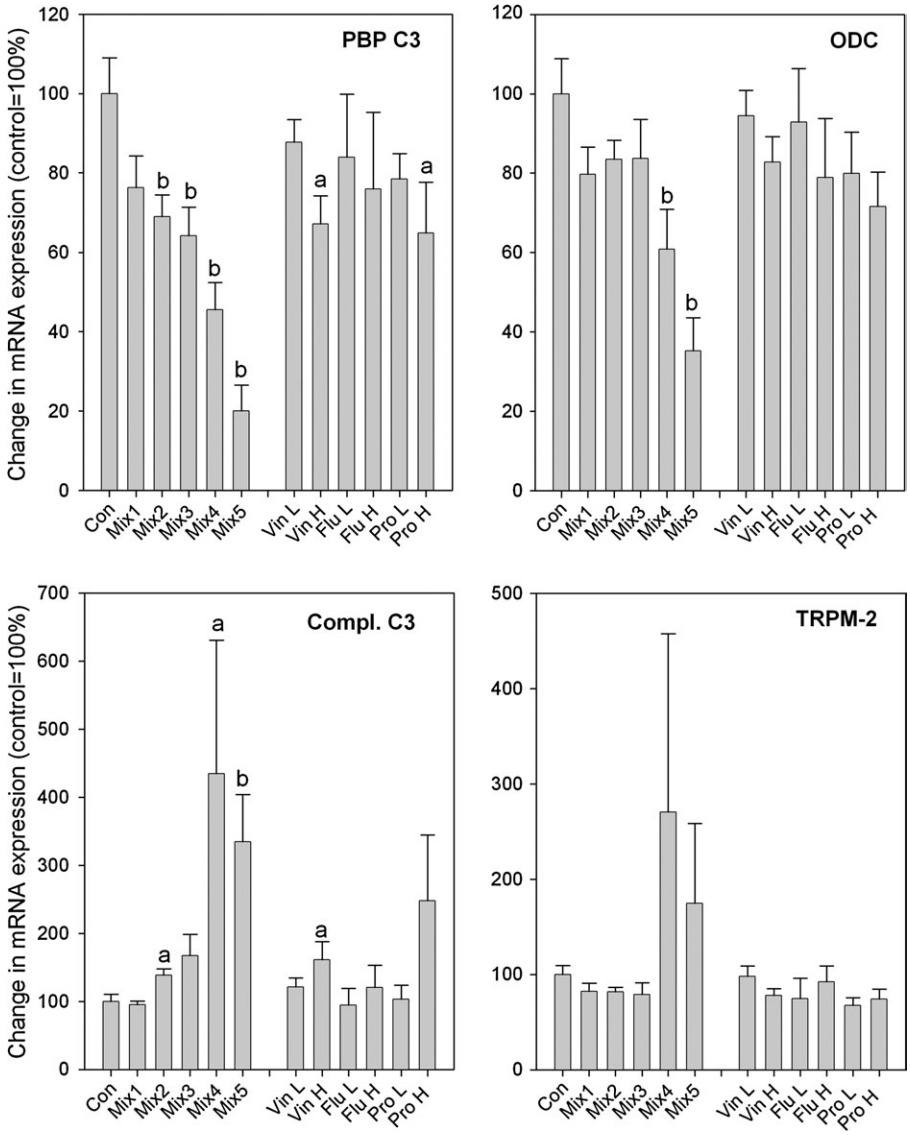

FIG. 4. The expression of *PBP C3*, *ODC*, *Compl.C3*, or *TRPM-2* mRNA in ventral prostates from male pups PND 16 is shown. Male pups were perinatally exposed to either vehicle, five doses of the mixture of vinclozolin (Vin), flutamide (Flu) and procymidone (Pro), or to low (L) or high (H) doses of the individual compounds. The mRNA expression was determined by real-time RT-PCR and calculated relative to the expression of the housekeeping gene *18S rRNA*. Controls were set to 100% and data represent means ± SEM. 'a' denotes a statistically significant difference compared to controls with $p < 0.05$ and 'b' denotes a significance with $p < 0.01$.

tissue architecture, receptor level, to changes at the gene expression level. This is surprising, considering that the features of the dose-addition concept lend themselves particularly to the modeling of events close to receptor binding and molecular activation processes. At this level of biological complexity, the basic premise of dose addition, i.e., that one compound can be replaced by a fraction of an equieffective dose of another chemical, is readily interpreted in terms of molecular interactions. Our results indicate that antiandrogen action involves effector chains that feed through to higher levels of biological complexity without violating the principles of dose addition. This insight may be of relevance for human and clinical studies and has the potential to be exploited in future biomonitoring studies.

Although the primary aim of our work was to assess the predictability of mixture effects of antiandrogens, the results of our study also allow assessments of the question as to whether there are joint effects when all mixture components are present at doses that individually do not induce detectable effects. This phenomenon, termed ''something from 'nothing''' (Silva *et al.*, 2002), has been observed with multicomponent mixtures of estrogenic agents in reporter-based assays (Rajapakse *et al.*, 2002; Silva *et al.*, 2002), the uterotrophic assay (Tinwell and Ashby, 2004), and vitellogenin induction in fish (Brian *et al.*,

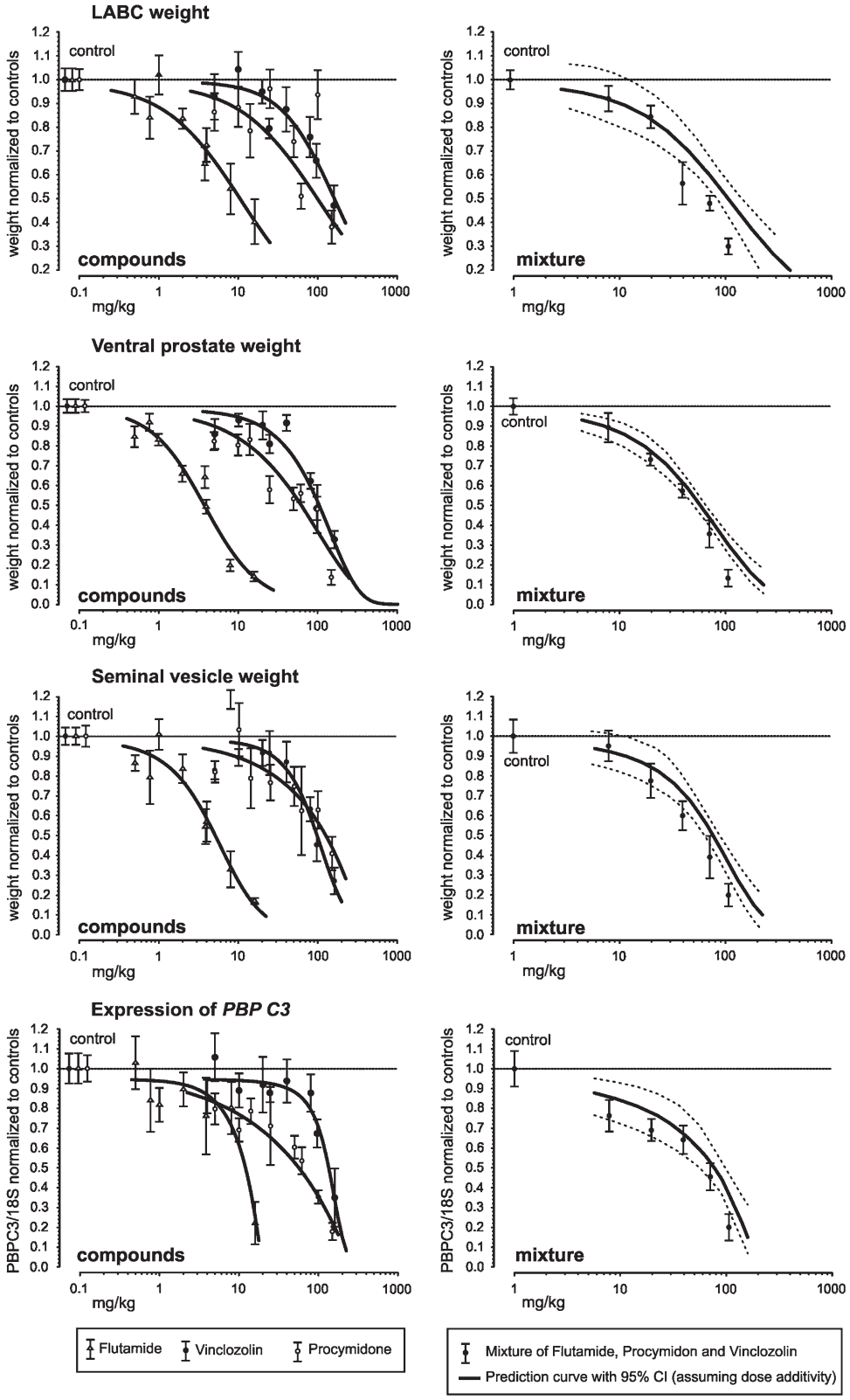

FIG. 5. Dose-response curves for the effects of low doses of vinclozolin, procymidone, and flutamide on weights of muscle LABC, seminal vesicles, and prostate and *PBP C3* gene expression in prostates (left column) in male pups PND 16. On the right side, the observed (data points) and predicted (solid line, with 95% confidence interval represented by broken lines) responses of the mixture assuming dose additivity are shown. All data were normalized to the mean control value (equaling 1).

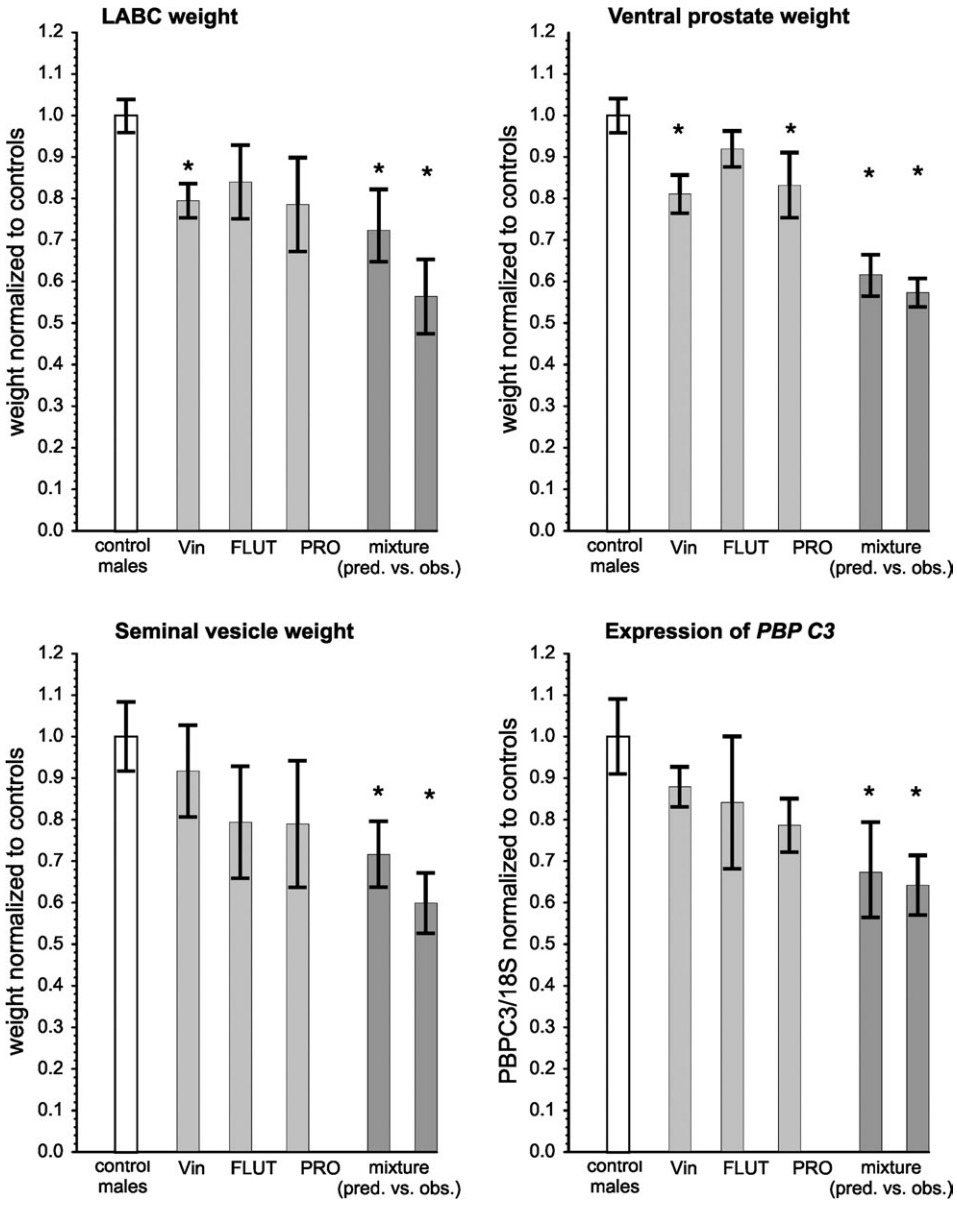

FIG. 6. Comparison of the observed effects at the low doses of the single compounds (vinclozolin 24.5 mg/kg, flutamide 0.77 mg/kg, procymidone 14.1 mg/kg) on the weights of muscle LABC (A), seminal vesicles (B), prostates (C), and *PBP C3* gene expression (D) with the effects of Mix 3, the mixture that combines the three chemicals at these low doses. The predicted effects of Mix 3 (dark bars) assuming additivity are shown as well. All data were normalized to the mean control value (equaling 1).

2005). The basis of this phenomenon derives from the theoretical assumptions that underlie the concept of dose addition: every agent at any dose contributes, in proportion to its toxic unit, to the overall effect of a mixture. Because every mixture component can be replaced totally or in part by an equal fraction of an equieffective dose of another, it does not matter whether the individual doses are also effective on their own. ''Something from 'nothing''' effects should occur even when individual toxicants are present at doses below effect thresholds, provided sufficiently large numbers of components sum up to a suitably high total effect dose. The results shown in Figure 6 support the idea that the ''something from 'nothing''' phenomenon also applies to the end points investigated in this study. A combination of the low doses of vinclozolin, flutamide, and procymidone induced an approximately 30 and 35% reduction of seminal vesicles weights and *PBP C3* expression, respectively. The effects induced by each chemical on its own did not reach statistical significance when compared with untreated controls, and thus these data support the ''something from 'nothing''' phenomenon. For the other end points, the Mix3 dose caused a more pronounced effect than that observed with the single chemicals. Generally, these

results show that lack of statistical significance cannot be equated with an absence of biological effects.

In conclusion, our results show that combinations of similarly acting antiandrogens are able to affect the male offspring of rats. These effects can be predicted fairly accurately on the basis of information about the potency of the individual mixture components by using the dose-addition concept. These data lend further support to the idea that antiandrogens act together to produce marked joint effects when combined at doses that individually produce small, statistically insignificant responses. The significance of these findings for human and environmental risk assessment must be emphasized; doses of endocrine active chemicals, which appear to exert only small effects when judged on their own, may induce marked responses when they act in concert with numerous, possibly unrecognized, similarly acting agents.

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Paper IV

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ORIGINAL ARTICLE

Combined exposure to anti-androgens causes markedly increased frequencies of hypospadias in the rat

S. Christiansen,* M. Scholze,† M. Axelstad,* J. Boberg,* A. Kortenkamp† and U. Hass*

*Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Søborg, Denmark, and †The School of Pharmacy, University of London, London, UK

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Correspondence:
Sofie Christiansen, Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
E-mail: sope@food.dtu.dk

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Summary

The incidence of hypospadias is increasing in young boys, but it remains unclear whether human exposure to endocrine disrupting chemicals plays a role. Risk assessment is based on estimation of no-observed-adverse-effect levels for single compounds, although humans are exposed to combinations of several anti-androgenic chemicals. In a mixture (MIX) study with three androgen receptor antagonists, vinclozolin, flutamide and procymidone, rats were gavaged during gestation and lactation with several doses of a MIX of the three chemicals or the chemicals alone. External malformations of the male reproductive organs were assessed on PND 47 using a score from 0 to 3 (normal to marked) for hypospadias. Markedly increased frequencies were observed after exposure to a MIX of the three chemicals compared to administration of the three chemicals alone. Anogenital distance at PND 1, nipple retention at PND 13, and dysgenesis score at PND 16 were highly correlated with the occurrence of hypospadias, and MIX effects were seen at doses where each of the individual chemicals caused no observable effects. Therefore, the results indicate that doses of anti-androgens, which appear to induce no hypospadias when judged on their own, may induce a very high frequency of hypospadias when they interact in concert with other anti-androgens.

Introduction

Studies have indicated that incidences of disorders in the male reproductive system, including hypospadias in newborn boys, have risen during the last 50 years (Toppari *et al.*, 2001), but the impact of human exposure to endocrine disrupting chemicals remains largely unknown at present. Animal studies have shown a clear connection between in utero exposure to endocrine disrupting chemicals and adverse effects on male reproduction (Foster, 2006; Gray *et al.*, 2006). Chemicals risk assessment is currently based on the no-observed-adverse-effect-levels for effects of single compounds. Using this approach, single endocrine disrupting chemicals alone may appear to be present in human tissues at levels too low to cause concern for adverse reproductive effects. However, as several anti-androgenic chemicals have been found as mixtures (MIX) in humans (Blount *et al.*, 2000; Swan *et al.*, 2005) including children (Brock *et al.*, 2002; Main *et al.*, 2006), it is crucial to elucidate the consequences of combined exposures to anti-androgens to assess human health risks.

We have studied the effects of a MIX of three androgen receptor (AR) antagonists, vinclozolin, flutamide (FLU), and procymidone, on male sexual differentiation in rats after exposure in utero and post-natally. Vinclozolin metabolites, procymidone and FLU compete with androgens for AR binding and suppress androgen-dependent gene transcription (Simard *et al.*, 1986; Kelce *et al.*, 1994, 1997; Ostby *et al.*, 1999). Common developmental effects of all three chemicals after exposure of male rats during development include reduced anogenital distance (AGD), nipple retention (NR), diminished prostate, testis and epididymal weights, and hypospadias (Gray *et al.*, 1994; Hib & Ponzio, 1995; Ostby *et al.*, 1999; Hellwig *et al.*, 2000; McIntyre *et al.*, 2001; Foster & McIntyre, 2002; Miyata *et al.*, 2002; Shimamura *et al.*, 2002).

The joint effects observed in the male offspring before weaning have been reported by Hass *et al.* (2007) and

Metzdorff *et al.* (2007) and were essentially dose-additive (Table 1). A combination of doses of each chemical that on its own did not produce significant effects, induced marked MIX effects on AGD, NR, seminal vesicles weight and histopathology, and PBP C3 gene expression in the prostate, and caused marked dysgenesis of external reproductive organs on PND 16.

In this paper, we broaden the range of endpoints relevant to anti-androgenic action with additional results from sexually mature male offspring. Dysgenesis of external reproductive organs was clearly seen at PND 16, but major events in the development of male external sex organs occur after this time during the pubertal period. Consequently, the aim was to assess the frequencies of hypospadias and other external sexual malformations in the young adult male rats. An additional aim was to examine if the anti-androgenic effects observed in the male pups were predictive biomarkers of external malformations observed in the young adult male rats later in life.

Materials and methods

Animals and exposure

Time-mated nulliparous, young adult Wistar rats (HanTac: WH; Taconic Europe, Ejby, Denmark) were supplied at day 3 (GD 3) of pregnancy.

Table 1 Summary of effects in male rat pups exposed to flutamide (FLU), vinclozolin (VIN), procymidone (PRO), or a mixture of FLU, vinclozolin, and procymidone (MIX) from GD 7 to PND 16. Based on Hass *et al.* (2007) and Metzdorff *et al.* (2007). Values are expressed as percent of control values for AGD and organ weights and as increase in number for nipple retention (NR) (control value = 0.0). Values for dysgenesis of external reproductive organs are shown as the increase in mean score (scores 0–3; control = 0) and the percentage of litters with a score larger than 1. Statistically significant effects compared to controls are shown in bold

Endpoint	FLU 0.77 mg/kg	VIN 24.5 mg/kg	PRO 14.1 mg/kg	MIX 39.3 mg/kg	Dose-additivity?	Joint effect compared to effect of single chemicals
AGD index, PND 1, % of control	95	97	99	**77**	Yes	Marked joint effect; no significant effect of single chemicals
NR, PND 13, number	**2.8**	**1.3**	**2.6**	**9.2**	Yes (slight synergy at high doses)	Marked joint effect; small significant effect of single chemicals
Right testis, PND 16, % of control	106	100	113	97	n.a	No joint effect; no significant effect of single chemicals
Epididymides, PND 16, % of control	**89**	**86**	**86**	**74**	n.a	Marked joint effect; significant effect of single chemicals
Ventral prostate, PND 16, % of control	91	**81**	**87**	**55**	Yes	Marked joint effect; no or small significant effect of single chemicals
Seminal vesicles, PND 16, % of control	75	85	81	**58**	Yes	Marked joint effect; no significant effect of single chemicals
LABC[#], PND 16, % of control	87	**81**	90	**57**	Yes	Marked joint effect; no or small significant effect of single chemicals
Bulbourethral glands, PND 16, % of control	80	**64**	72	**28**	n.a	Marked joint effect; no significant effects of FLU and PRO, but clear significant effect of VIN
PBP C3 expression in prostate, PND 16, % of control	81	96	83	**65**	Yes	Marked joint effect; no significant effect of single chemicals
Dysgenesis of external reproductive organs, PND 16, mean score	**0.6**	**0.5**	**0.5**	**1.8**	n.a	Marked joint effect; small significant effect of single chemicals
Dysgenesis of external reproductive organs, PND 16, % with score > 1	0	0	0	**54**	n.a	Marked joint effect; no significant effect of single chemicals

[#]Levator ani/bulbocavernosus muscles; n.a: not analysed.

The substances used were corn oil (vehicle), vinclozolin (VIN) and procymidone (PRO) (all from Bie & Berntsen, Herlev, Denmark), and FLU (Sigma Aldrich, Brøndby, Denmark).

Two selected doses of the three chemicals were applied in parallel with the MIX experiment. The composition of the MIX was chosen according to the equi-effective doses of each individual component that produced a half-maximal NR (MIX ratio 31 : 1 : 18, VIN: FLU: PRO). This step was taken to avoid that one single chemical contributed disproportionately to the overall MIX effect. The animals were dosed by gavage from GD 7–21 and from PND 1–16. On GD 4, the dams were distributed into groups of animals with similar body weight distributions (16 animals per group in control, MIX-doses of 7.9, 19.7, 39.3 and 70.8 mg/kg, VIN-doses of 24.5 and 95.9 mg/kg; 8 animals per group in MIX-dose of 106.2 mg/kg, FLU-doses of 0.8 and 3.9 mg/kg, PRO-doses of 14.1 and 61.8 mg/kg). The smaller group size for the highest MIX group, FLU and PRO was selected, because the animals in these groups were only to be used for the assessment of endpoints during lactation, while those with group size 16 were designated for assessment of effects in adult offspring too. One randomly chosen male pup per litter was kept after weaning from these litters.

To compensate for lack of single compounds data from PRO and FLU in this study, results from groups of 10–12 litters exposed to PRO (25, 50 and 100 mg/kg) were included from another study in our lab (data not published). The animals were exposed at GD 7-PND 16 and malformations were scored at 8 month of age. Results from groups of 10–12 male rats (representing about 5 litters) exposed to FLU (2.5, 10 and 100 mg/kg) were included from the literature (Miyata *et al.*, 2002). In that study, rats (Sprague–Dawley) were exposed from GD 14 to PND 3 and malformations in male rats were scored at PND 60.

The animals were treated humanely and with regard for alleviation of suffering. The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

Malformation score on PND 47

Malformations and variations in the external male reproductive organs including cleft phallus/hypospadias and blind vaginal opening were scored on PND 47 by the same technician who was blinded with respect to exposure group.

The changes were scored on a scale from 0 to 3 using the following criteria:

Score 0: Normal external reproductive organs.

Score 1: Slight cleft or variations of preputium, but no hypospadias or vaginal opening.

Score 2: Clear hypospadias, but no vaginal opening.

Score 3: Marked hypospadias with exposure of the os penis and vaginal opening.

When scoring the PRO exposed rats at 8 month of age, the number of rats with hypospadias was counted, whereas FLU exposed rats in Miyata *et al.* (2002) was presented as percentage hypospadias (Miyata *et al.*, 2002).

Statistics

The statistical means for AGD and NR (group mean) were estimated by using a generalized non-linear mixed modelling approach (Vonesh & Chinchilli, 1996), with litter as a random effect modifier for individual effect data (see Hass *et al.*, 2007 for more details). Scores of external genitalia on PND 16 and PND 47 are responses that can take values from a number of categories (multinomial data). They were assumed to follow a multinomial distribution and analysed by using a cumulative logit model (McCullagh & Nelder, 1989): the most likely occurrence of each score is estimated in dependence of the dose (likelihood). For malformation data on PND 47 all four scores were used (Fig. 2), and to maintain comparability with external data (Fig. 1), additionally they were categorized into a binary variable, with scores 0 and 1 (no hypospadias) vs. scores 2 and 3 (clear and marked hypospadias). In cases where only one score was observed in the sample, the missing data variation prevented the calculation of confidence intervals. All analyses were carried out using the SAS procedure PROC GENMOD (SAS version 8; SAS Institute Inc, Cary, NC, USA).

Results

Table 1 shows the effects of a MIX of VIN, PRO and FLU, and of all three chemicals individually, on sexual differentiation in male rat offspring from PND 1 to PND 16 as reported in Hass *et al.* (2007) and Metzdorff *et al.* (2007). The MIX showed dose additivity when evaluated in terms of AGD index, NR, accessory sex organ weights (ventral prostate, seminal vesicles, and LABC), as well as PBP C3 gene expression in the prostate at PND 16. Here, we describe effects that were observed later in male offspring.

Malformations in adult male rats

The two highest MIX doses (39.3 and 70.8 mg/kg) and VIN on its own (95.9 mg/kg) produced significant increases in malformations at PND 47. More than 50% of the animals showed clear signs of hypospadias; at the

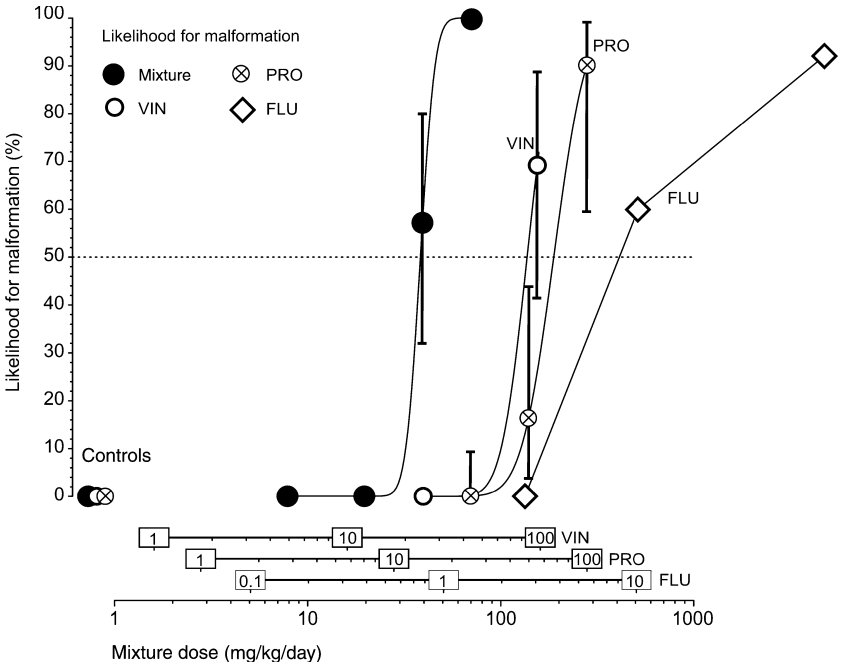

Figure 1 Malformations in male rats exposed to a mixture of three anti-androgens. The observed mixture effects for malformation in adult male rats, characterized by their likelihood (black dots), 95% confidence belt (shown as error lines) and regression fit (black line), are compared with the expected dose response curves of the individual compounds (symbols with corresponding curves), at the doses present in the mixture. To enable comparisons with the effects of the combination, the curves of the individual chemicals were scaled to the levels present in the mixture. Data for procymidone are from eight month old male rats, data for flutamide are from (Miyata *et al.*, 2002). The likelihood for malformations presented here are for clear and marked malformations (scores 2 and 3).

MIX dose of 39.3 mg/kg, around 56% (range 30–80%) of them can be expected to have this malformation with a statistical 95% certainty (Fig. 1). This MIX dose contained 24.5 mg/kg VIN. However, when VIN was tested on its own at this dose, none of the animals exhibited signs of malformations (score of 0). The 39.3 mg/kg MIX dose also contained 14.1 mg/kg PRO and 0.77 mg/kg FLU. In a previous study, we tested PRO on its own, but signs of malformations were not detected at a dose of 25 mg/kg. Although FLU-induced malformations at PND 47 were not tested in our lab, results communicated by Miyata *et al.* (2002) showed that FLU was without observable effects in Sprague–Dawley rats at the higher dose of 2.5 mg/kg. Thus, when tested individually at doses present in the MIX, none of the compounds showed any signs of malformations. Yet, when combined (MIX dose of 39.3 mg/kg), the malformation frequency (clear or marked hypospadias) was significantly increased to 60% ($p < 0.01$).

Correlations between malformation scores at PND 47, and AGD, NR and dysgenesis score at PND 16

To assess whether early biomarkers of disruption of sexual differentiation can be used to predict the occurrence of hypospadias in later life (PND 47), we correlated anti-androgenic endpoints in the pups, i.e. AGD, NR and the dysgenesis score at PND 16, to the mean likelihood for malformations at PND 47 (Fig. 2a–c). The data (Fig. 2) are based on group means, i.e. the mean responses were estimated for all doses and controls and then compared. Figure 2a shows that if the male rats show an average 50% reduction of the AGD-index at PND 1, it is most likely that after 6 weeks, a majority of them (72%) will have at least a slight malformation at PND 47, and 25% will even reveal marked hypospadias. If they exhibit on average six nipples at PND 13, more than 20% of them will present with slight malformations at PND 47, with 10% having a clear hypospadias (Fig. 2b). Comparison of the scores for dysgenesis at PND 16 with those for malformations at PND 47 (Fig. 2c) revealed a clear correlation between the corresponding likelihoods. Severe signs of dysgenesis at PND 16 in 50% of the male rats are strongly predictive of marked hypospadias at PND 47 in nearly all of these animals. However, signs of weak dysgenesis at PND 16 (score 1) do not seem to be a good early biomarker for later hypospadias, at least when the number of affected rats is small.

Discussion

Our study has shown clearly that anti-androgens with a similar mode of action (AR antagonism) act together in an additive way for a broad spectrum of endpoints assessed in rat pups before weaning (Table 1, Hass *et al.*, 2007; Metzdorff *et al.*, 2007). These findings are consistent with the previously reported additivity of anti-androgens on other endpoints such as AR activation in vitro and in vivo (Nellemann *et al.*, 2003; Birkhøj *et al.*, 2004) and with the cumulative effects of DBP and DEHP and of linuron and butyl benzyl phthalate reported by (Howdeshell *et al.*, 2007; Hotchkiss *et al.*, 2004).

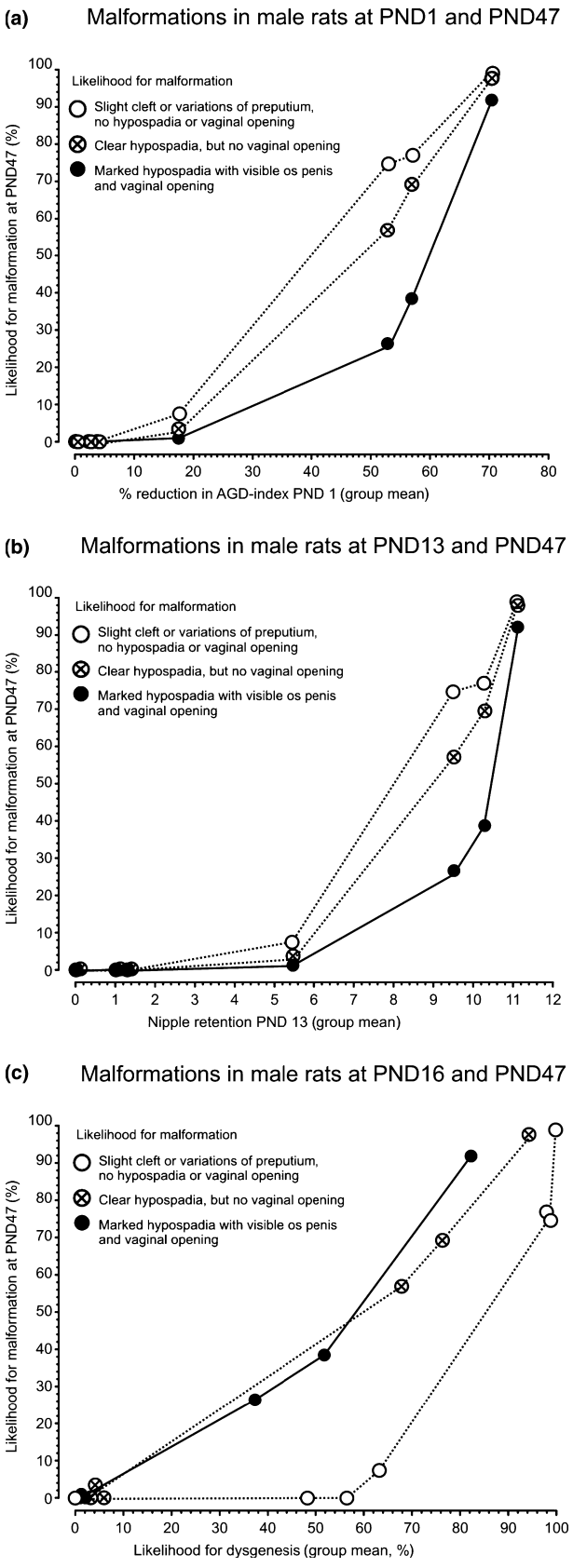

Figure 2 (a–c). Relationships between early anti-androgenic effects and malformations in male rats at PND 47. Shown are combination effects of vinclozolin, flutamide and procymidone, characterized by their likelihoods for three malformation scores, in relation to a % reduction in AGD-index (a) (control male = 0%, female = 100%), an average nipple retention at PND 13 (b) and the likelihood for dysgenesis at PND 16 (c). Group means are derived from this study with four mixture doses of three anti-androgens and two doses of vinclozolin.

There is good evidence that combined administration of anti-androgens may lead to marked effects on AGD, NR, reproductive organ weights, *PBP C3* gene expression in the prostate and pronounced dysgenesis of external reproductive organs at PND 16, even when the components are present at levels below doses associated with observable effects (Hass *et al.*, 2007; Metzdorff *et al.*, 2007). This phenomenon has also been observed with multi-component MIXs of estrogenic agents in reporter-based assays (Rajapakse *et al.*, 2002; Silva *et al.*, 2002), the uterotrophic assay (Tinwell & Ashby, 2004), and vitellogenin induction in fish (Brian *et al.*, 2005).

The results in Fig. 1 indicate that similar low dose MIX effects also apply to malformations (hypospadias) in adults that are the consequence of disruption of male sexual differentiation during development. A combination of 24.5 mg/kg/day VIN, 0.77 mg/kg/day FLU and 14.1 mg/kg/day PRO induced hypospadias in 56% of the male offspring, whereas the frequency after exposure to 24.5 mg/kg VIN alone was similar to the untreated controls (0%).

Unfortunately, the frequencies of hypospadias on PND 47 induced by FLU and PRO individually at the dose levels present in the 39.3 mg/kg MIX exposure were not investigated directly in the same study, because animals from these dose groups were not kept after weaning. We have bridged this gap by making comparisons with historical data on PRO from our own lab, and with literature reports about FLU (Miyata *et al.*, 2002). Even at a dose considerably higher (25 mg/kg) than present in the MIX (14.1 mg/kg), PRO did not produce observable malformations at PND 47. Similarly, FLU lacked the ability to cause such effects at 2.5 mg/kg (Miyata *et al.*, 2002), a dose approximately three times higher than that administered in the MIX (0.77 mg/kg). Thus, we have good reason to believe that the frequencies of hypospadias produced by PRO and FLU individually at the levels present in the MIX would have been 0% at the doses applied in the MIX. This line of argumentation is further supported by considering the similarities in the mode of action of three anti-androgens, which led to quite similar anti-androgenic effects in the pups (Table 1, Hass *et al.*, 2007; Metzdorff *et al.*, 2007). The MIX effects seen here

clearly exceed predictions of independent action, because 56% is much higher than the sum of 0% + 0% + 0%.

In studies with single chemicals (finasteride and linuron), it has been shown earlier that changes in AGD (Bowman *et al.*, 2003) and NR (McIntyre *et al.*, 2002) are highly predictive of permanent malformations of the male reproductive system. Here, we demonstrate that early effects in pups that arose from exposure to MIXs of anti-androgens are also good predictors of the malformations observable later in adult and sexually mature male rats (Fig. 2a–c).

In conclusion, our results show that doses of anti-androgens, which induced no hypospadias when judged on their own, may cause a very high frequency of hypospadias when they interact in concert with other similarly acting anti-androgens. The significance of these findings for human and environmental risk assessment must be emphasized, because they clearly indicate that risk assessment based on NOAELs for single anti-androgens alone underestimates the risk for hypospadias and other adverse anti-androgenic effects.

In addition, changes in the early biomarkers; AGD, NR and dysgenesis PND 16 could predict malformations in the adult male rat. This should be taken into account in future risk assessments of individual chemicals and MIXs.

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Paper V

Christiansen S, Scholze M, Dalgaard M, Vinggaard AM, Axelstad M, Kortenkamp A & Hass U. Synergistic disruption of external male sex organ development by a mixture of four anti-androgens. *Submitted to Environmental Health Perspectives*

Synergistic disruption of external male sex organ development by a mixture of four anti-androgens

Sofie Christiansen[1^)], Martin Scholze[2^)], Majken Dalgaard [1)], Anne Marie Vinggaard[1)], Marta Axelstad [1)], Andreas Kortenkamp [2*)], and Ulla Hass [1*)]

1) National Food Institute, Technical University of Denmark
Dept. of Toxicology and Risk Assessment
Mørkhøj Bygade 19
DK-2860 Søborg
Denmark
Tel.: +45 72 34 75 44

2) The School of Pharmacy
University of London
29-39 Brunswick Square
London WC1N 1AX
United Kingdom
Tel.: +44 20 7753 5908

*) Correspondence regarding reproductive toxicity should be addressed to Ulla Hass, fax number +45 72347699, email address ulha@food.dtu.dk, communications regarding mixture effect assessments to Andreas Kortenkamp, fax number +44 20 77535811, email address andreas.kortenkamp@pharmacy.ac.uk

^ Both authors contributed equally.

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Disclaimers, competing interests

There are no competing interests to declare.

Short running head

Synergisms with a mixture of four antiandrogens

Key words

Cumulative effects, combination effects, mixtures, dose addition, independent action, anti-androgens, male sexual differentiation, phthalates, azole fungicides, DEHP, vinclozolin, finasteride, prochloraz

Abbreviations

AGD	Anogenital distance
AR	Androgen receptor
DEHP	di(2-ethylhexyl) phthalate
DHT	Dihydrotestosterone
ECHA	European Chemicals Agency
FIN	Finasteride
GD	Gestational day
LABC	levator ani/bulbocavernosus muscles
NOAEL	No-observed-adverse-effect-level
NR	Nipple retention
NR NOAEL	No-observed-adverse-effect-level based on nipple retention
PND	Postnatal day
PZ	Prochloraz
U.S EPA	United States Environmental Protection Agency
VZ	Vinclozolin

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Abstract

Background: By disrupting the action of androgens during gestation, certain chemicals present in food, consumer products and the environment can induce irreversible demasculinisation and malformations of sex organs among male offspring. However, the consequences of simultaneous exposure to such chemicals are not well described, especially when they exert their actions by differing molecular mechanisms.

Objectives: To fill this gap, we investigated the effects of mixtures of a widely used plasticizer, di(2-ethylhexyl) phthalate (DEHP), two fungicides present in food, vinclozolin and prochloraz, and a pharmaceutical, finasteride, on landmarks of male sexual development in the rat, including changes in anogenital distance, retained nipples, sex organ weights and malformations of genitalia. These chemicals were chosen because they disrupt androgen action according to differing mechanisms of action.

Results: Strikingly, the effect of combined exposure to the selected chemicals on malformations of external sex organs was synergistic, and the observed responses were greater than would be predicted from the toxicities of the individual chemicals. In relation to other hallmarks of disrupted male sexual development, including changes in anogenital distance, retained nipples, and sex organ weights, the combined effects were dose additive. When the four chemicals were combined at doses equal to no-observed-adverse-effect levels estimated for nipple retention, significant reductions in anogenital distance were observed in male offspring.

Conclusions: Since unhindered androgen action is essential for human male development in foetal life, these findings are highly relevant to human risk assessment. Evaluations

that ignore the possibility of combination effects may lead to considerable underestimations of risks associated with exposures to chemicals that disrupt male sexual differentiation.

Introduction

Certain chemicals present in consumer products and food can disrupt the action of steroidal androgens in foetal life, with irreversible consequences for later life stages. In the male rat, exposure to such chemicals during development leads to incomplete masculinisation and severe malformations of the reproductive organs (Christiansen et al. 2008; Welsh et al. 2008; Wilson et al. 2008). Because of their relevance to humans, the effects seen in laboratory animals have led to considerable concerns. Substances of interest, so-called anti-androgens, include certain phthalates, widely used as plasticizers, a few pharmaceutical agents and some fungicides. Phthalates have attracted significant recent interest, with several regulatory bodies currently engaged in risk assessments (U.S.EPA, ECHA). As yet, these regulatory efforts have taken little account of realistic human exposures which is to several phthalates and other anti-androgens simultaneously (Blount et al. 2000; Brock et al. 2002; Swan et al. 2005; Main et al. 2006; Marsee et al. 2006). With the aim of exploring the effects of combined exposure to anti-androgens during gestation, we have conducted large developmental toxicity mixture experiments in the rat with the widely used phthalate di(2-ethylhexyl) phthalate (DEHP), the fungicides vinclozolin and prochloraz, present in certain food items, and the pharmaceutical finasteride.

These anti-androgens were selected because they can disrupt male sexual differentiation in different ways, by a variety of mechanisms. In foetal life, testosterone is a key driver of the differentiation of the Wolffian duct system into the vas deferens, epididymis, seminal vesicles and external genitalia. Phthalates with a certain ester side-chain length,

such as DEHP, can lower testosterone levels by interfering with the uptake of cholesterol precursors into foetal Leydig cells, where testicular androgen production takes place (Foster et al. 1980). In the rat, malformations of internal reproductive organs (epididymis, testes) are the consequence. Because dihydrotestosterone (DHT) is derived from testosterone through enzymatic conversion by 5α-reductase, lower testosterone concentrations also affect the development of tissues that rely on DHT (prostate and external genitalia). DHT is further required for the regression of nipple anlagen in male rats and for the growth of the perineum to produce the normal male anogenital distance (AGD) which is longer than in females (Imperato-McGinley et al. 1985; Imperato-McGinley et al. 1986). Due to reduced DHT levels in the wake of suppressed testosterone synthesis, retained nipples and feminised AGDs are also seen in male rats exposed to phthalates in foetal life (Carruthers and Foster 2005; Jarfelt et al. 2005; Foster 2006). Vinclozolin metabolites impact more directly on the development of DHT-dependent tissues by antagonising the androgen receptor (AR) (Gray et al. 1999b; Wolf et al. 2000; Hass et al. 2007). In disrupting the enzymatic conversion of testosterone to DHT through inhibition of 5α-reductase (Clark et al. 1990; Bowman et al. 2003), the pharmaceutical finasteride induces an effect spectrum similar to AR antagonists. Finally, the fungicide prochloraz disrupts androgen action by inhibiting the conversion of progesterone to testosterone and by antagonising the AR (Vinggaard et al. 2002; Noriega et al. 2005; Vinggaard et al. 2005; Laier et al. 2006; Blystone et al. 2007).

One goal of mixture toxicology is to anticipate the toxicities of untested mixtures of chemicals when only the effects of individual components are known (Könemann 1980; Könemann 1981; Hermens et al. 1984; Hermens et al. 1985). A second issue of relevance

to regulatory toxicology concerns the question of mixture effects at low doses. A view that has persisted over the last 30 years is that combination effects do not occur when each chemical is present at doses equal to their no-observed-adverse-effect-levels (NOAELs), or lower (COT 2002; VKM 2008). This is thought to be the case with chemicals that act through different mechanisms, but not with agents that target similar molecular structures. The NOAEL is the highest tested dose at which no statistically or biologically adverse effects can be identified and is used in regulatory toxicology as a point of departure for establishing "acceptable" exposures for humans.

In experiments with rats, the combined effects of anti-androgens that act through similar mechanisms, such as several phthalates that suppress testosterone synthesis (Howdeshell et al. 2008) or some AR antagonists (Hass et al. 2007; Metzdorff et al. 2007; Christiansen et al. 2008) were additive and could be predicted well from the effects of the individual mixture components. In addition, combination effects were observed at NOAELs for the individual chemicals. Less obvious is how combinations of anti-androgens behave that disrupts androgen action via different mechanisms. Here, we have investigated the predictability of the combined effects of mixtures composed of dissimilarly acting anti-androgens. In pursuing this aim, we decided to combine all chemicals in the mixture in proportion to their NOAELs for effects on male sexual development. This was done in order to investigate whether combination effects occur at individual doses of the compounds that are of regulatory relevance, and in order to avoid the testing situation that one or two chemicals contributed disproportionately to an overall combination effect. Our motivation was not to model environmental intakes. A second goal was to test whether combination effects occur at doses around the NOAELs for the single chemicals,

and this also forced us to combine the antiandrogens in proportion to their relative potency, rather than their environmental exposures.

Materials and Methods

Chemicals

VZ (vinclozolin), CAS No. 50471-44-8, purity 99%, FIN (finasteride), CAS No.98319-26-7, purity 99.8%, DEHP (di-(2-ethylhexyl)phthalate), CAS No. 117-81-7, purity 99% and PZ (prochloraz), CAS No. 67747, purity 99.6%, were obtained from VWR - Bie & Berntsen (Herlev, Denmark). The chemicals were dissolved in corn oil (used as vehicle) from VWR - Bie & Berntsen.

Animals and dosing

Time-mated nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark, body weight approximately 200 g) were supplied at day 3 of pregnancy. The day following mating was designated gestational day (GD) 1, and postnatal day (PND) 0 was the day of birth. On the day after arrival at our facilities (GD 4), the dams were randomly distributed into groups of 16 or 8 animals with similar body weight (bw) distributions. A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) was provided *ad libitum*.

Test chemicals and mixtures were administered by gavage from GD 7 to the day before expected birth (GD 21), and from PND 1 until PND 16 to the dams (see also Fig. 1), in a dosing volume of 2 ml/kg bw. The dose levels and group sizes are shown in Table 1. The studies were performed using 4 blocks (with one week in between), and all dose groups were equally represented in the blocks.

Studies and dose levels

Before the mixture experiment was conducted, dose-response studies for each individual chemical were carried out. The dose ranges were chosen with the aim to cover the entire range of responses, from no apparent effects to complete feminisation, as determined by measurements of AGD and NR. The same approach was used in our first mixture study described in Hass et al 2007 (Hass et al. 2007). The dose levels selected for the dose-response studies were based on the reductions of AGD and increases of NR reported for vinclozolin (Gray et al. 1999b; Gray et al. 1994; Hellwig et al. 2000; Shimamura et al. 2002; Hotchkiss et al. 2002), finasteride (Clark et al. 1990; Hib and Ponzio 1995; Bowman et al. 2003), DEHP (Gray et al. 1999a; Gray et al. 2000; Jarfelt et al. 2005) and prochloraz (Laier et al. 2006). In order to gain information about the variability of effects between studies as well as to facilitate a direct comparison within the mixture study, selected doses of vinclozolin, finasteride, DEHP and prochloraz were run in parallel with the mixture experiment.

Two or three selected doses of the four chemicals were administered in parallel with the mixture doses. For the mixture experiment, a master mixture was prepared by combining doses of the four chemicals at a fixed ratio of 500:1:300:500 (vinclozolin: finasteride: DEHP: prochloraz). Thus, the master mixture contained 15,000 mg vinclozolin, 30.0 mg finasteride, 9,000 mg DEHP, and 15,000 mg prochloraz in 600 ml corn oil. The master mixture was administered undiluted in a dosing volume of 2 ml/kg bw to achieve the highest mixture dose of 130.1 mg/kg/day. Dilutions of 1 : 1 and 1 : 9 in corn oil were prepared for the two remaining dose groups of 65.05 mg/kg/day and 13.01 mg/kg/day, respectively. The lowest mixture (mix1) dose was equivalent to the sum of the chemicals'

individual NOAELs for retained nipples (NR NOAEL), the middle dose (mix2) was 5-fold higher, and the highest dose (mix3) 10-fold this values. The animals were treated humanely and with regard for alleviation of suffering. The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

Determination of changes in male sexual differentiation

Anogenital distance and nipple retention

In all studies, AGD and NR were recorded by the same technician who was blinded with respect to exposure groups. After birth, all live pups in the litter were weighted, sexed and AGD measured using a stereomicroscope. The crude AGD measurements were transformed to the AGD-index, by dividing AGD by the cube root of body weight. The cube root was used, because this converts a three-dimensional endpoint (weight) into a one-dimensional such as the AGD (Gallavan et al. 1999; Gray et al. 1999a). This ratio assumes that the relationship between AGD and transformed body weight is directly proportional and linear. In order to assess the validity of this assumption, we explored the influence of using the transformed body weight as a co-variable in statistical analyses. However, relevant differences between these two approaches were not detected. In the interest of keeping the model parameters as simple as possible (to avoid over-parameterisation), we decided to base all statistical analyses on the AGD-index.

The body weights of all pups were recorded on PND 13, together with the number of nipples/areolas, defined as a dark focal area (with or without a nipple bud) located where

nipples are normally present in female offspring. Females normally have 12 nipples, but may in a few cases show up to 14.

Organ weights and assessment of malformations in external genitalia

On PND 16, 2-6 male pups per litter were weighed and their external genitalia were inspected. From one male per litter, the following organs were excised and weighed: testis, epididymis, ventral prostate, seminal vesicles, levator ani/bulbocavernosus muscles (LABC), bulbourethral glands, adrenals, kidney, and liver. In all examined males the level of demasculinisation was scored on a scale from 0 to 3, with the observer being blinded with respect to dose group. The scores were as follows:

Score 0 (no effect): Normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact. In the perineal area, thick fur extended caudally from the base of the genital tubercle and half the distance to the anus. A furless area circumscribed the anus.

Score 1 (mild dysgenesis of the external genitals): A small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening was observed, estimated 0.5-1.4 on an arbitrary scale. The size of the genital tubercle was decreased. The furless area around the anus expanded towards the base of the genital tubercle, but thick fur was still present at the base of the genital tubercle.

Score 2 (moderate dysgenesis of the external genitals): The preputial cleft was larger, estimated 1.5-2.4 on an arbitrary scale. The urethral opening was situated half-way down towards the base of the genital tubercle (hypospadia). Partly furless areas or thin fur was

noted in the perineal area extending from the base of the genital tubercle and caudally to the furless area circumscribing the anus.

Score 3 (severe dysgenesis of the external genitals): The preputial cleft was large, estimated 2.5-3.5 on an arbitrary scale. The urethral opening was situated further than half-way down the inferior side of the genital tubercle to the base of the genital tubercle (hypospadia). At the base of the genital tubercle a groove extending laterally was observed (similar to control females at PND 16). The male rat was totally furless in the entire perineal area.

For data analysis, we simplified the scoring into a binary variable, with scores 0 and 1 (no cleft phallus) vs. scores 2 and 3 (clear and marked cleft phallus).

One male per litter was kept after weaning in order to examine the external reproductive organs after sexual maturation. On PND 47 malformations and changes of the external male reproductive organs including cleft phallus/hypospadia and blind vaginal opening were scored by the same technician, again blinded with respect to exposure groups. The changes were scored on a scale from 0 to 3 using the following criteria:

Score 0: Normal external reproductive organs.

Score 1: Slight cleft or variations of preputium, but no hypospadia or blind vaginal opening.

Score 2: Clear hypospadia, but no blind vaginal opening.

Score 3: Marked hypospadia with visible os penis and blind vaginal opening.

For data analysis, we transformed the scoring into a binary variable, with scores 0 and 1 (no hypospadias) vs. scores 2 and 3 (clear and marked hypospadias).

Data normalisations

To enable pooling of the data from our studies, which in some cases stretched over a period of three years, all regression analyses had to be based on normalised values. For organ weight data from PND 16, a linear relationship between organ weights and body weight was found, based on all control pups, with the corresponding regression lines going through the origin within their 95% confidence belts. Therefore, individual organ weights were normalized to the ratio between that organ and body weight. Furthermore, slight differences in control values between studies were controlled for by standardizing these ratios to 1 by dividing them through the mean normalised organ weight from unexposed male pups. In a similar way, we standardized the absolute AGD indices to relative values between 1 (no effect on male AGD index) and zero (complete feminisation). The mean AGD indices from unexposed male and female pups were used to define the minimum and maximum responses, respectively. Assessment of nipple retention yielded count values which were used without further normalisation. Data normalisations were also not necessary in the case of the binary malformation scores.

Dose-response analyses

By using Shapiro-Wilk's and Bartlett's tests, we confirmed that all continuous effect measurements were normally distributed and homogeneous. Responses different from controls were assessed for statistical significance by multiple testing methods (global error rate α= 5%, two-sided). Statistical analyses were always adjusted for litter effects,

by using litter as an independent, random and nested factor, and statistical significance was then assessed on the basis of multiple contrast tests (see (Hass et al. 2007) for more details). When organ weights were analysed, body weight was included as covariate.

Statistical dose response regression analyses were conducted in the same way for the mixture and the single compounds, with analyses for the single compounds based on pooled effect data from initial dose-response studies and the repeated doses that were run concurrently with the mixture study (Supplementary Table 1a, b). A best-fit approach (Scholze et al. 2001) was used, where various non-linear regression models (logit, probit Weibull, generalized logit) were fitted independently to the same data set. The best fitting model was selected on the basis of a statistical goodness-of-fit criterion (information criterion of Schwarz). To control for litter effects, dose-response data were analysed by using a generalized non-linear mixed modelling approach (Vonesh and Chinchilli 1996), with litter as a random effect modifier for individual effect data. The dose-response functions in Scholze *et al*. (Scholze et al. 2001) assume monotonic increasing relationships, but some endpoints studied here yielded monotonic decreasing forms. To maintain compatibility with the dose-response functions described by Scholze et al., the upper and lower asymptotes in the regression models were exchanged, i.e. the θ_{max} in the corresponding functions of Table 2 were used as θ_{min} and vice versa.

All analyses were carried out using the SAS procedure PROC GENMOD, PROC MIXED and PROC MULTTEST (SAS version 8, SAS Institute Inc, Cary, NC, USA). More details can be found in (Hass et al. 2007; Metzdorff et al. 2007; Christiansen et al. 2008).

Calculation of mixture effect predictions

In making predictions for the combined effects of DEHP, vinclozolin, prochloraz and finasteride, we assumed that each chemical in the mixture did not exacerbate or diminish the effects of the other components. Mixture effects according to such "no interaction" or "additivity" assumptions can be calculated by using two alternative concepts, dose addition and independent action. Dose addition looks at mixture effects in terms of a "dilution principle". It assumes that one chemical can be replaced totally or in part by an equal fraction of an equi-effective dose of another, without diminishing the overall combined effect (Loewe and Muischnek 1926). Dose addition is often used for mixtures composed of chemicals that act through a similar or common mode of action (U.S.EPA 1986; U.S.EPA 2000; COT 2002; U.S.EPA 2002). Its application to the present mixture of four anti-androgens appeared justified because all chemicals affect the biological action of testosterone and DHT during development, and produce common effect outcomes. However, it can be argued with equal justification that the similarity assumption characteristic for dose addition is not applicable to the chosen mixture because its component chemicals produce their effects by a diversity of molecular mechanisms. For this reason, we also employed the alternative concept of independent action to construct additivity expectations. Independent action assumes that the joint effects of a combination of agents can be calculated by adopting the statistical concept of independent events (Bliss 1939). It is viewed as appropriate for mixtures of chemicals with diverse modes of action (COT 2002), however, dose addition and independent action can yield very similar additive mixture effect predictions at low doses.

Under the assumption of dose additive combination effects, or those conforming with independent action, dose-response relationships for the mixture with defined mixture ratio ("fixed mixture ratio design (Faust et al. 2001)) were predicted using the best-fit dose-response regression curves of the individual compounds (Supplementary Table 1a-c) and compared to the observed mixture effects (Table 3). Equation 1 allows the calculation of any effect dose of a mixture under the hypothesis of dose additivity. For this, the dose response functions of all mixture components and the mixture ratios have to be known:

$$\mathrm{EDx}_{\mathrm{mixture}} = \left(\frac{p_1}{\mathrm{EDx}_1} + \frac{p_2}{\mathrm{EDx}_2} + \frac{p_3}{\mathrm{EDx}_3} + \frac{p_4}{\mathrm{EDx}_4} \right)^{-1}. \qquad (1).$$

Here, EDx_1, EDx_2, EDx_3 and EDx_4 are the effect doses of vinclozolin, finasteride, DEHP and prochloraz that on their own produce the same quantitative effect x as the mixture, and p_1, p_2, p_3 and p_4 are the relative proportions of the corresponding individual doses present in the total mixture dose. The individual effect doses were derived from the dose response functions for the compounds by using their inverse functional form. If no effects were observed in the tested dose ranges for the i^{th} compound, we replaced the missing information about the effect doses in equation 1 (EDx_i) by assuming either a maximal effect (realised mathematically by approximating a "Yes/No" step function through a dose-response logit function with model steepness parameter θ_2=40) or no effect at all (i.e. setting the ratio p_i/EDx_i in equation 1 to zero) for doses exceeding those that were actually tested. This procedure had to be adopted in order to calculate mixture effect predictions for genital malformations, because two of the mixture components, DEHP and prochloraz, were without observable effects on their own (see Supplementary Tables

1a and 1c. These two extrapolation scenarios define the only possible prediction range of mixture responses according to equation 1.

The basic version of independent action has been formulated under the simple assumption that the susceptibilities of the individuals of an at-risk-population to different dissimilarly acting mixture compounds are not correlated with each other (Bliss 1939). For a four-component mixture this is can be defined by the equation

$$E(c_{mix}) = 1 - \left(1 - E(c_1)\right) \bullet \left(1 - E(c_2)\right) \bullet \left(1 - E(c_3)\right) \bullet \left(1 - E(c_4)\right) \quad . \qquad (2)$$

Here, $E(c_1)$, $E(c_2)$, $E(c_3)$, and $E(c_4)$ denote the fractional effects (x %) caused by the individual concentrations c_1, c_2, c_3, and c_4 of vinclozolin, finasteride, DEHP and prochloraz, respectively, and $E(c_{mix})$ is the total effect of the mixture concentration c_{mix}. The individual effects of mixture compounds $E(c_i)$ are estimated from the concentration response functions determined for single substances (Table 1 main text). Eq. 2 cannot be transformed into an explicit term for an effect mixture dose EDx_{mix}, but the value of EDx_{mix} satisfying the equation for a given effect level x has to be computed by an iterative procedure. The statistical uncertainty for the predicted mixture effects and effect doses was determined by using the bootstrap method (Efron and Tibshirani 1993) and expressed as 95 % confidence limits for the predicted mean estimate. Differences between predicted and observed effect doses were deemed statistically significant when the 95 % confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

Results

Dose-response analyses with the individual mixture components

Of all indicators of disrupted sexual development, alterations in retained nipples were the most sensitive endpoint, with effects becoming noticeable at the lowest doses. Changes in anogenital distance were almost as sensitive, followed by reductions in prostate and LABC weights and genital malformations. Finasteride was by far the most potent chemical. It exhibited dose-response curves with very shallow gradients for all endpoints. Vinclozolin was active in a narrower dose range, with correspondingly steeper dose-response curves, and the characteristics of prochloraz and DEHP fell between these extremes. Information about details of the dosing schedules and the timing of the various studies can be found in Table 1. A summary of dose-response descriptors for all tested chemicals, based on the pooled outcome of studies conducted before the mixture experiment, is given in Supplementary Tables 1a-c; responses seen with individual chemicals tested in parallel with the mixture are described in Supplementary Table 2.

Prediction of mixture effects

The mixture ratio used in our study was chosen to reflect the NOAELs of each chemical alone, judged in relation to the most sensitive indicator of disruption of male sexual development, increased nipple retention (NR). These NOAELs, here referred to as NR NOAELs, were estimated in our laboratory before the mixture experiment began (3 mg/kg/day for DEHP, 5 mg/kg/day for vinclozolin, 5 mg/kg/day for prochloraz and 0.01

mg/kg/day for finasteride). Our NR NOAELs agree well with the doses that are currently used as starting points for establishing tolerable exposures for humans (Supplementary Table 3). Mixture doses equal to the sum of NR NOAELs (13.01 mg/kg/day), and multiples of 5 (65.05 mg/kg/day) and 10 (130.1 mg/kg/day) were tested experimentally.

Additive mixture effects

Figure 2 shows how the experimentally observed responses compared with the additive effects of the mixture that were predicted by using dose addition. The effect outcomes of the mixture experiment are listed in Supplementary Table 2. Changes in anogenital distance, retained nipples, prostate weights and weights of LABC were chosen as the endpoints for evaluation. In all cases, the overall effect means measured for all litters came close to the predicted response curves. There was considerable overlap between the mean responses for individual litters and the anticipated effects. To evaluate the relationship between predictions and observations statistically, we used the effect variability seen with the single chemicals to estimate statistical confidence limits for the predicted (dose additive) effect doses by applying boot-strapping methodology. A statistically significant difference between anticipated and observed effect dose could not be detected, except for doses that caused changes in anogenital distance at an effect level of 60% change relative to controls (Table 3). The mixture effect doses predicted by using the alternative concept of independent action did not differ significantly from those anticipated by dose addition. For all endpoints, the observed mixture effects were stronger than the responses attributable to any one single mixture component. Our data suggest that the combined effects of DEHP, vinclozolin, prochloraz and finasteride are

additive when the evaluation is based on changes in anogenital distance, retained nipples, prostate weights and weights of LABC.

Synergistic effects for genital malformations

Strikingly, a completely different picture emerged when we evaluated dysgenesis of the external genitalia in young male rats at PND 16 and PND 47. Administration of the mixture led to severe malformations at PND 16 characterised by enlarged preputial clefts, and urethral openings located towards the base of the genital tubercle (not at its top, as normally expected), similar to hypospadias in humans. A laterally extending groove resembling a vaginal opening was often found at the base of the genital tubercle. The number of males affected by these abnormalities at PND 16 was scored and expressed as likelihood for severe malformations (Supplementary Table 2, Fig. 3). Milder signs of genital dysgenesis, such as small cavities appearing on the inferior side of the genital tubercle, minor clefts in the preputial opening or small genital tubercles, were not taken into account. At the lowest tested mixture dose, none of the effects classed as severe malformations were noted. At the second highest mixture dose, 26% of the animals were affected, but at the highest tested mixture dose virtually all animals (94%) showed severe malformations. Similar malformation rates for hypospadias, including exposure of the os penis and blind vaginal opening became apparent at PND 47. At the doses included in the mixture, none of the chemicals alone induced malformations, with the exception of vinclozolin at 50 mg/kg/day, where a low frequency was observed (Supplementary Table 2).

Based on the dose-response data for the individual chemicals (pooled data from previous studies and tests conducted concurrently with the mixture experiment), we calculated the probability for malformations at PND 16 according to dose addition and independent action. We found that both these concepts underestimated the observed effects by a considerable margin (Fig. 3). Because DEHP and prochloraz on their own were without detectable effects at doses between 3 and 30 mg/kg/day, and 5 and 50 mg/kg/day, respectively, we based the calculation of combination effects according to dose addition on two conjectures: We either assumed that DEHP and prochloraz did not contribute to the overall combination effect, or that their individual effects approached 100% as the doses increased beyond the tested range. These two assumptions define the extremes of the dose addition prediction area shown in Figure 3. The independent action prediction yielded a curve that was congruent with the high-dose extreme of the dose addition prediction area. Malformations occurred at three-fold lower mixture doses than foreseen by both these models (Fig. 3, Table 3). Because of the steepness of the underlying dose-response curve, this meant that a dose anticipated as being without effect produced malformations in 94% of the animals. Evaluated in relation to the low dose margin of the dose addition prediction area, the mixture caused malformations at two-fold lower doses. The experimentally observed responses clearly exceeded the predictions, suggesting that the combined effect of DEHP, vinclozolin, prochloraz and finasteride is synergistic with respect to genital malformations.

Combination effects at doses around NOAELs

Next, we tested the idea that mixture effects should not occur when mixed-mode chemicals are combined at dose levels close to those that are used as points of departure in regulatory toxicology for the estimation of safe human exposures, such as NOAELs. DEHP, vinclozolin, prochloraz and finasteride were combined at doses equal to their individual NR NOAELs for changes in nipple retention that were estimated in studies preceding the mixture experiment. At these doses, none of the individual chemicals on their own produced changes in anogenital distance, yet the combination induced a statistically significant effect, well predicted by dose addition and independent action. At 10-fold higher doses, the effects of vinclozolin and finasteride alone (but not of DEHP and prochloraz) reached statistical significance. At this point, the combination strongly exacerbated the response, with anogenital distances half-way between those expected in normal males and normal females (Fig. 4 A).

With weight changes of the prostate and LABC as the endpoints for assessment, the NR NOAEL combination did not produce effects distinguishable from untreated controls. When tested in parallel with the mixture, finasteride on its own yielded statistically significant responses for nipple retention at its previously estimated NR NOAEL of 0.01 mg/kg/day. This prevented us from conducting the above analyses in relation to retained nipples.

Combined at their NR NOAELs, the four anti-androgens did not provoke appreciably elevated malformation rates, but the 5-fold higher mixture dose induced malformations in

around 25% of the males. At this point, none of the single chemicals alone is likely to have led to observable malformations (Supplementary Table 2, Fig. 3). Even at the levels present in the 10-fold higher mixture dose, three of the single chemicals alone did not induce discernable effects, but vinclozolin on its own caused malformations in about 5% of the affected males. Yet, when combined at the doses equivalent to 10-fold NR NOAELs, malformations in nearly all male offspring were seen (Fig. 4 B). Similar results became apparent when malformations were analysed later in the rats lives, at PND 47 (Fig. 4 C).

Discussion

In relation to certain sensitive hallmarks of disrupted male sexual differentiation, dose addition and independent action proved to be equally useful tools for the prediction of combination effects of mixed-mode anti-androgens. For alterations in anogenital distance, retained nipples and prostate and LABC weight changes, reasonable anticipations of mixture effects could be achieved on the basis of dose-response descriptors for the individual mixture components. That this occurred despite the different mechanistic premises that underlie the two prediction concepts is coincidental. It is not explained by toxicological features, but by the mathematical algorithms behind each concept (Drescher and Bödeker 1995). Our results hold the promise that predictions of combination effects could be obtained in the future without conducting mixture experiments, by employing modelling approaches. Considering the high cost and long duration of reproductive toxicity studies, this might greatly aid efforts in regulatory toxicology.

All the more remarkable are our observations of synergistic interactions with genital malformations such as hypospadias. Because the affected males derived from dams that were dosed in the same way as those where additive effects on characteristics of disrupted sexual development were found, dosing errors cannot account for the deviations from expected additivity. Assessment errors introduced during the scoring of the malformations, leading to the detection of false positives, are also not a likely explanation, since we disregarded the milder forms of malformations which may be

difficult to diagnose. The fact that finasteride, when tested concurrently with the mixture, proved to be more effective in inducing retained nipples than seen previously also does not explain the synergism. There were no changes in the chemicals' effectiveness of causing malformations. As with the other endpoints used in this study, androgens are key factors for the normal development of the penis from the genital tubercle. The observed malformations, including hypospadias, are indicative of disruption of androgen action. This leads to a failure of the folding and fusion that needs to take place during development to form normal genitals. Judged from this viewpoint, the mixture would be expected to induce additive effects on malformations too. Although we are unable to offer a mechanistic explanation for the synergisms, it seems that genital development in the rat is also governed by events that differ in important details from those operating during the regression of nipple anlagen and the formation of normal AGD. Genital tubercle development generally also includes embryonic processes requiring embryonic cell movements and apposition of the two advancing tissues (Gupta and Goldman 1986).

Recent studies suggest a previously unidentified role for the progesterone receptor, possibly interacting with the androgen receptor, in disturbed genital tubercle development (Willingham et al. 2006). In utero exposure to natural or synthetic progesterones can increase the risk of hypospadias in male mice and intake during pregnancy in humans has been associated with increased risks of hypospadias (Carmichael et al. 2005; Willingham et al. 2006). Prochloraz, one of the chemicals in the mixture, was able to induce increased testicular progesterone concentrations in male rat foetuses, and this effect occurred at lower dose levels than those present in the mixture investigated here (Vinggaard et al. 2005; Laier et al. 2006; Blystone et al. 2007). Thus, elevated progesterone levels due to

prochloraz exposure may have a role in the synergisms with severe hypospadias that we observed in our study. Generally, synergisms may be caused by toxicokinetic interactions, where due to interference with uptake or metabolism one or several mixture components are present at levels higher than would occur if these chemicals were given individually. However, we regard toxicokinetic interactions as an unlikely explanation, because dose-additivity was found for all other hallmarks of male sexual disruption, including endpoints assessed around or at the same developmental age as the genital malformations.

Our findings echo the observations made by Rider and colleagues (Rider et al. 2008) where a mixture of butyl benzyl phthalate, di-*n*-butyl phthalate, DEHP, vinclozolin, procymidone, linuron, and prochloraz induced frequencies of hypospadias that exceeded those predicted by dose addition. As in our study, other endpoints that were investigated simultaneously revealed dose-additive effects. Rider et al. used historical data for the calculation of additivity expectations. Because these data were sometimes based on dosing regimens that differed from those used in the mixture experiments, there is a degree of uncertainty as to whether the excess malformations in that study represent a true synergism. Our evaluations are based on dose-response data for single chemicals and mixtures from one large study, and therefore substantiate concerns about the synergistic induction of genital malformations by mixed-mode of action anti-androgens.

The effects seen in the rat are of high relevance to the human and deserve serious consideration in ongoing risk assessment efforts. Very recently, changes in anogenital

distance (Swan et al. 2005) and suppressions of androgen synthesis have been associated with anti-androgen exposure in humans (Main et al. 2006).

A point that requires careful consideration is whether additive or even synergistic effects between antiandrogens are also likely to occur at low, environmentally relevant exposure levels. Human intakes of DEHP alone are estimated to be about 1000 times lower than used in our study (NRC 2008), and similar low exposures can be expected for the other chemicals that we used. It is clear that our developmental rat model would not have produced any responses, had we combined all mixture components at such low levels. However, human populations are not just exposed to four antiandrogens, but to a larger, although poorly defined number of chemicals with a potential to interfere with androgen action. Under dose addition, theory predicts that all these chemicals, even at low levels, act together to produce combined effects (Kortenkamp et al. 2007). Whether this effect will become observable in human populations, depends on the number of chemicals, their levels and the sensitivity of humans relative to the rat.

Conclusions

Our findings contradict the widely held view (COT 2002; VKM 2008) that chemical mixtures exhibiting varied modes of action are without combination effects at levels near supposed dose thresholds, used in regulatory toxicology as points of departure for the derivation of tolerable human exposures. Were this assumption correct, we should not have been able to observe alterations in anogenital distance when all four chemicals were combined at their NR NOAELs. Despite the synergism which we detected, the NR NOAEL combination did not produce appreciably elevated malformation rates, but this may be due to the lower sensitivity of this endpoint. It is likely that a mixture composed of a larger number of anti-androgens would have produced malformations at correspondingly lower doses, approaching NR NOAELs. Our observations show that the use of NOAELs as points of departure in risk assessment may lead to underestimations of risk when exposure is to several anti-androgens with common effect outcomes, regardless of mechanism. This highlights the deficiencies of the predominant chemical-by-chemical approach in risk assessment. It is necessary to supplant current procedures by moves towards taking account of mixture effects, and our results underline the importance of such a paradigm change.

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Tables

Table 1. Studies, groups, doses and number of time-mated animals per group

Study (No.)	Groups and doses	No. mated per group (pregnant)
(1) Vinclozolin dose-response and prochloraz range finding	Control: vehicle-dosed	16 (13)
	Vinclozolin: 5, 10, 20, 40, 80 or 160 mg/kg/day	8 (6-8)
	Prochloraz; 50 or 150 mg/kg/day	8 (6-8)
(2) Finasteride and DEHP, dose-response	Control: vehicle-dosed	16 (15)
	Finasteride: 0.001, 0.01, 0.1, 1, 10 or 100[a]mg/kg/day	8/10 (7-9)
	DEHP: 10, 30, 100, 300, 600 or 900 mg/kg/day	8(6-8)
(3) Prochloraz and DEHP, dose-response	Control: vehicle-dosed	16(15)
	Prochloraz: 5, 10, 25, 50, or 100 mg/kg/day	8(5-8)
	DEHP: 3, 10, 30, or 100 mg/kg/day	16/8(6-8/14)
(4) Mixture study of vinclozolin, finasteride, DEHP and prochloraz, Mixture ratio 5: 0.01:3: 5	Control: vehicle-dosed	16 (13)
	Mixture: 13.01, 65.05 or 130.10 mg/kg/day	16 (11-16)
	Vinclozolin: 5[b] or 50[d] mg/kg/day	8 (7-8)
	Finasteride: 0.01[b] or 0.1[d] mg/kg/day	8 (6)
	DEHP: 3[b], 15[c] or 30[d] mg/kg/day	8 (5-7)
	Prochloraz: 5[b], 25[c] or 50[d] mg/kg/day	8 (6-7)

[a] This dose of finasteride induced perinatal death and low pup weight and had to be decreased to 50 mg/kg bw/day from PND1-3 (block 1) and from GD 18 (block 2), from GD 11 (block 3), and in block 4 the dams received 50 mg/kg bw/day during the whole dosing period. 50mg/kg bw/day was used for the data analysis [b] Dose of single chemical present in the 13.01 mg/kg bw/day mixture dose. [c] Dose of the single chemical included in the 65.05 mg/kg mixture dose. [d] Dose of the single chemical included in the 130.10 mg/kg mixture dose.

Table 2. Statistical uncertainty of predicted and observed effect doses for the mixture of DEHP, vinclozolin, prochloraz and finasteride.

Effect level	Effect doses for the mixture [mg/kg/day]					
	Observed [a]		Predicted by DA [b]		Predicted by IA [c]	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Relative AGD index						
90 %	11.8	[4.3;34.7]	12.2	[0.3;53.8]	12.3	[0.2;58.2]
60 %	77.1	[59.4;99.8]	**195.6**	**[147.2;262.3]**	**214.3**	**[179.2;250.6]**
Number of nipples/areolas						
3	10.6	[6.5;15.3]	8.8	[5.6;13.6]	6.7	[4.1;13.5]
6	23.3	[17.0;29.4]	25.8	[16.8;35.7]	25.1	[16.8;41.4]
Organ weights in relation to the controls						
Ventral Prostate (mg)						
90%	44.7	[13.9;79.3]	44.6	[6.2;65.2]	24.4	[0.1;68.6]
60%	103.7	[78.9;130.5]	162.1	[128.6;226.5]	148.4	[98.4;200.8]
LABC[d] (mg)						
90%	51.0	[19.3;81.9]	48.1	[3.5;78.3]	25.9	[0.02;93.1]
60%	118.9	[93.9;152.1]	179.7	[142.8;325.3]	176.2	[109.1;252.8]
Likelihood for malformation at PND16 (cleft phallus)						
10%	25.5	[21.0;31.2]	**96.5 – 133.1[e]**		**140.0 – 140.1 [e]**	
50%	37.5	[32.6;44.6]	**149.1 – 226.2 [e]**		**226.2 – 226.4 [e]**	

All predicted effect doses statistically significantly different from the observed effect doses are shown in bold;
[a] Effect doses as calculated from the dose response functions given in Table 2a;b
[b] DA – dose addition, predicted effect doses are based on pooled data from studies for vinclozolin, prochloraz, finasteride and DEHP and were calculated from the respective dose response functions given in Table 2;
[c] IA – independent action, predicted effect doses are based on pooled data from studies for vinclozolin, prochloraz, finasteride and DEHP and were calculated from the respective dose response functions given in Table 2;
[d]: LABC – Levator ani/bulbocavernosus muscles;
[e]: No pups with malformations were observed for the tested doses of DEHP and prochloraz. For this reason, the calculation of expected mixture effect was based on two conjectures: For doses exceeding the tested dose range, we assumed that effects were absent, or that they reached maximal response. These two worst-case extrapolations define the only possible range of effects and were used to calculate the predicted effect doses for the mixture.

Legends to Figures

Figure 1

Outline of the study design adopted for dose-response analyses of anti-androgens and their mixtures.

Mated dams (orange arrow) were dosed from gestational day (GD) 7 to postnatal day (PND) 16 (green bar). Male offspring (brown arrow) were examined for multiple signs of disrupted sexual development, including anogenital distance (AGD) on PND 1, retained nipples (PND 13), weights of prostate and levator ani/ bulbocavernosus muscles (LABC) (PND 16) and malformations of the genital organs (PND 16, 47).

Figure 2

Prediction and assessment of combination effects of anti-androgens with mixed modes of action on hallmarks of male sexual development in the rat.

Multiple doses of a combination of DEHP, vinclozolin, prochloraz and finasteride with a mixture ratio of 3: 5: 5: 0.01 were administered to dams throughout gestation. Their male offspring were investigated for **A**, changes in anogenital index at PND 1, **B**, weight changes of the levator ani/ bulbocavernosus muscles (LABC) at PND 16, normalised in relation to body weights and untreated controls, **C**, number of retained nipples at PND 13 and **D**, weight changes of ventral prostates at PND 16, normalised to body weight and untreated controls. Black circles are means of individual litters, and red circles the group means of all investigated litters, with their standard errors (red error bars). The predicted

mixture effects (green solid curves) were derived by using dose addition, with their 95% confidence belts (broken green lines). Total mixture doses are given.

Figure 3

Synergistic effects on induction of genital malformations.

Combinations of DEHP, vinclozolin, prochloraz and finasteride (mixture ratio as in Fig. 2) were given to dams. Their male offspring was evaluated for the likelihood of induction of genital malformations at PND 16, including enlarged preputial clefts, and urethral openings located towards the base of the genital tubercle, similar to hypospadias in humans. Black dots are the mean likelihood of these malformations, based on 10-15 litters per group, with their 95% confidence intervals. The blue solid line is the best-fit regression model (Weibull) with 95% confidence belt (broken blue lines). The green curves marking the edges of the green shaded area are the lower and upper estimates of combination effects according to dose addition. The grey curve overlaying the upper bound of the dose addition prediction area depicts the combined effects predicted by using independent action. Total mixture doses are given.

Figure 4

Combination effects at low doses of DEHP, vinclozolin, prochloraz and finasteride.

Dams were dosed with 5 mg/kg/d vinclozolin (VZ), 0.01 mg/kg/d finasteride (FIN), 5 mg/kg/d prochloraz (PZ) and 3 mg/kg/d DEHP. These doses are the NOAELs estimated for nipple retention (NR NOAEL). Male offspring were evaluated for **A,** changes in anogenital

distance, **B**, genital malformations at PND 16, and **C**, genital malformations at PND 47. Grey bars show results in control males and females, red bars are the responses seen after administration of each chemical alone. The red bars labelled "mixture" are the observed effects of the combination of individual NR NOAELs (left panels), or those seen after administration of a 10-fold higher dose (right panels). The green bars are predicted dose additive effects (DA), blue bars those anticipated according to independent action (IA). Error bars are 95% confidence intervals, stars denote statistically significant effects compared to controls.

Figure 1

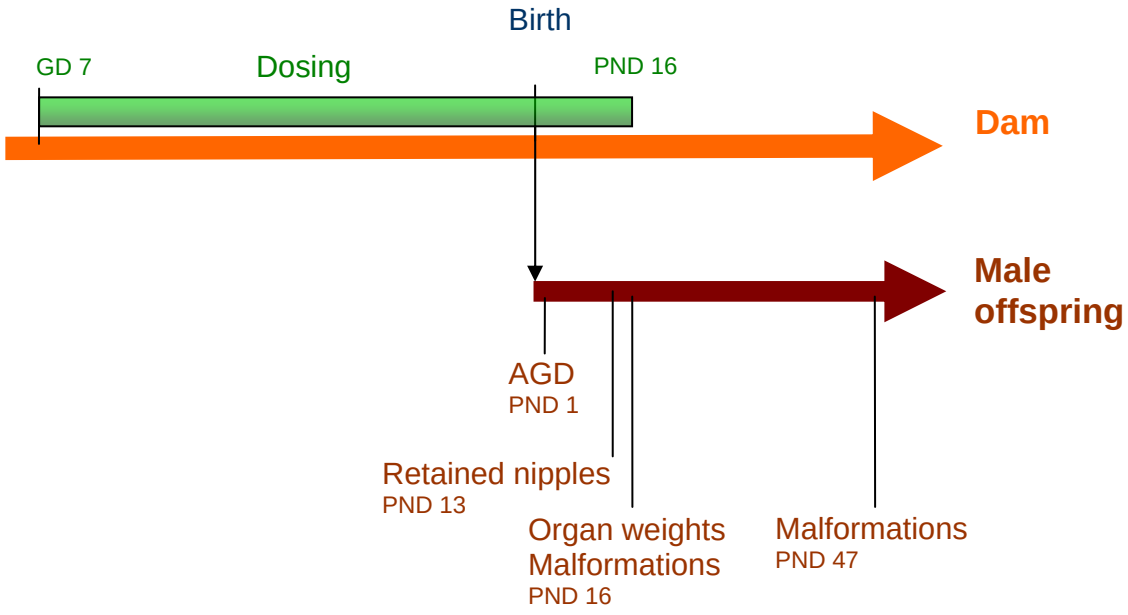

Birth
GD 7
Dosing
PND 16
Dam
Male offspring
AGD
PND 1
Retained nipples
PND 13
Organ weights
Malformations
PND 16
Malformations
PND 47

Figure 2

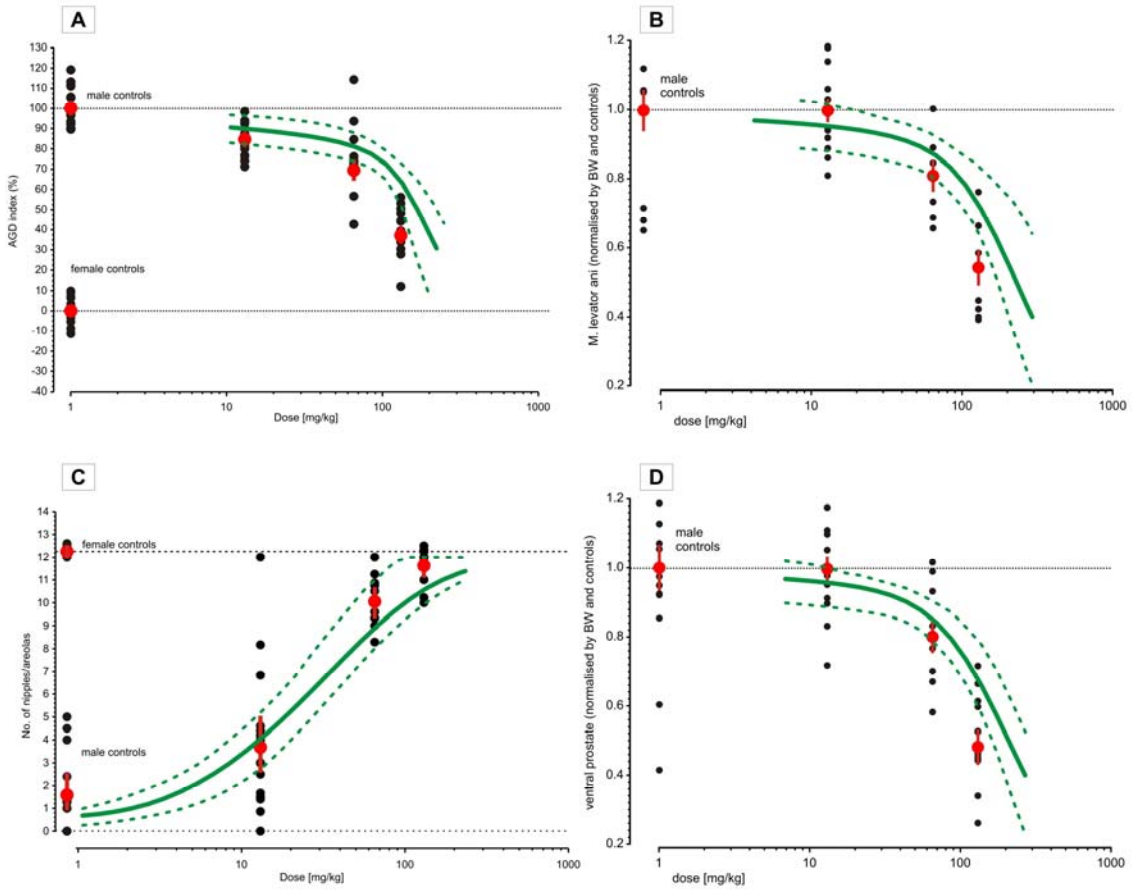

A
male controls
female controls
AGD index (%)
Dose [mg/kg]
B
male controls
M. levator ani (normalised by BW and controls)
dose [mg/kg]
C
female controls
male controls
No. of nipples/areolas
Dose [mg/kg]
D
male controls
ventral prostate (normalised by BW and controls)
dose [mg/kg]

Figure 3

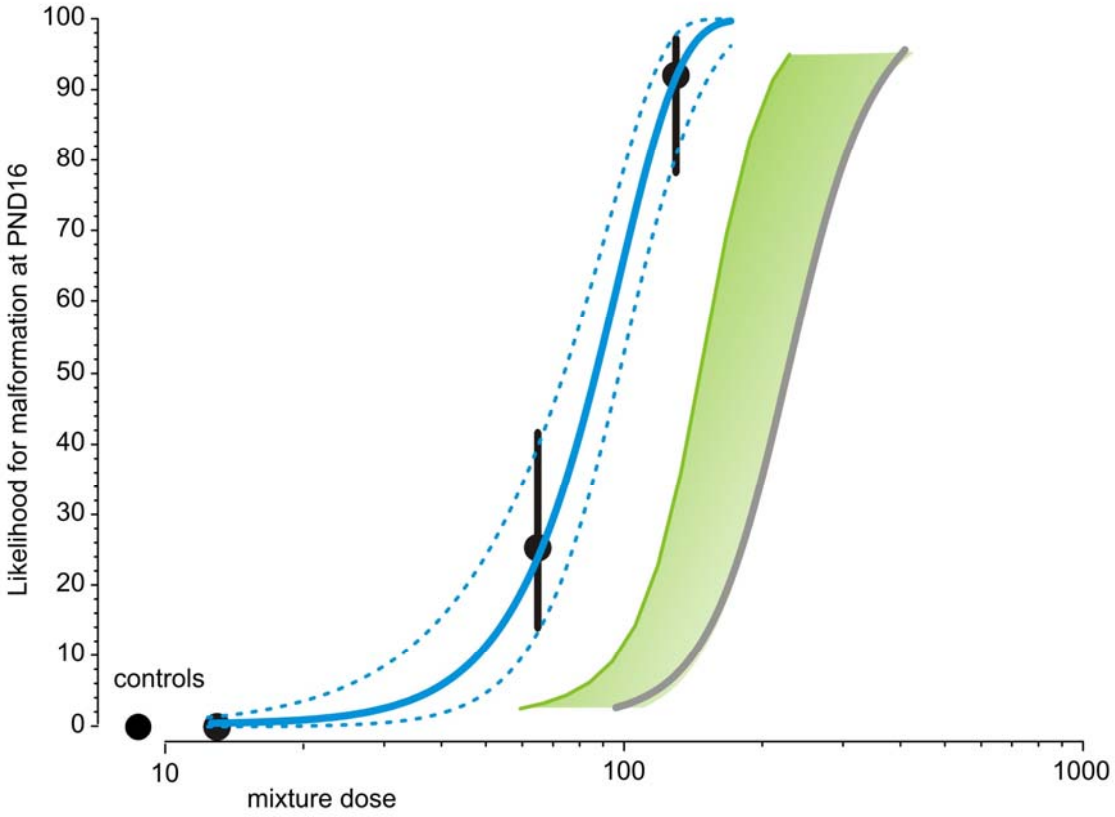
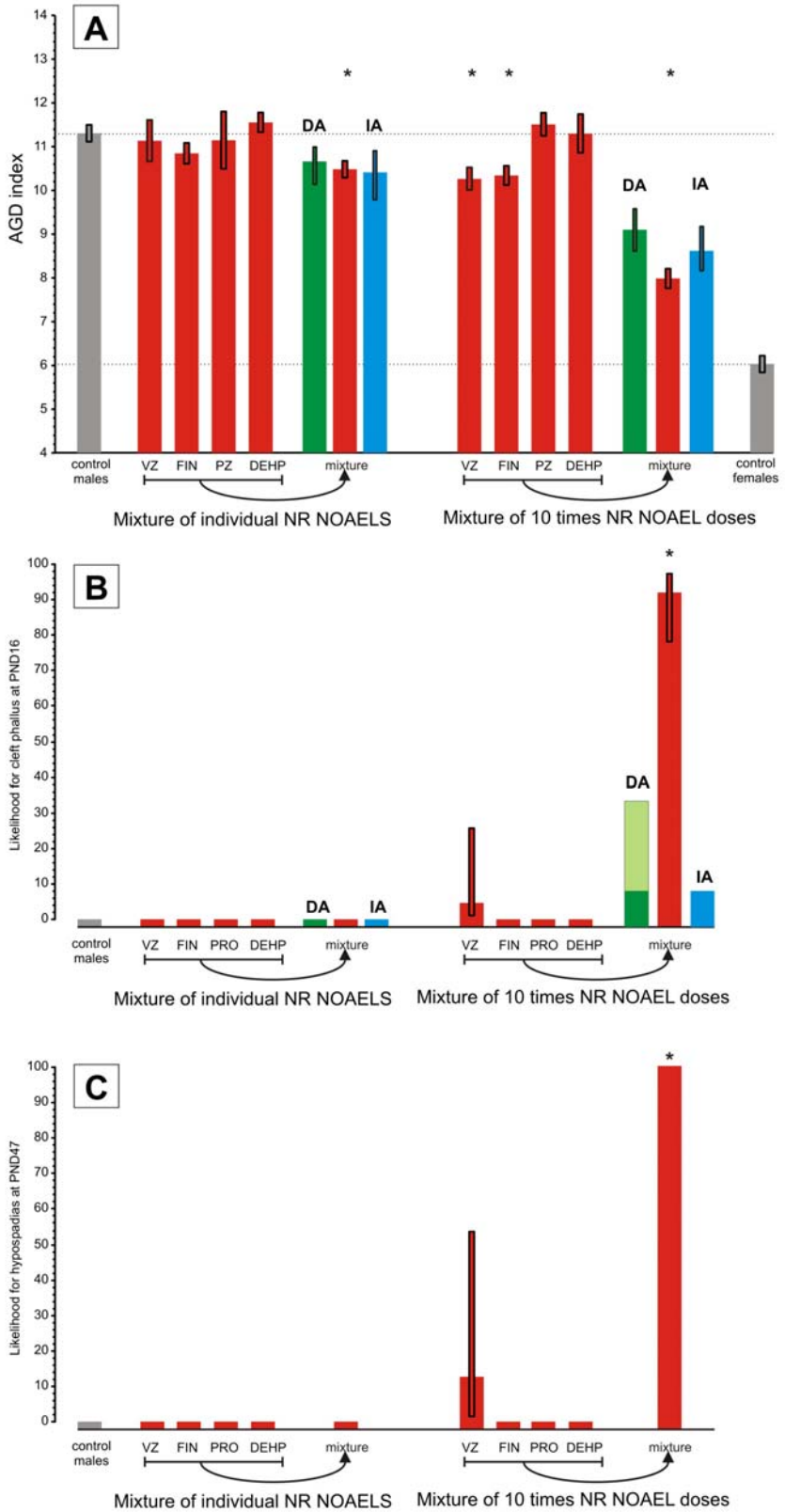

Likelihood for malformation at PND16
100
90
80
70
60
50
40
30
20
10
0
controls
10
100
1000
mixture dose

Figure 4


A
AGD index
DA
IA
control males
VZ FIN PZ DEHP
mixture
control females
Mixture of individual NR NOAELS
Mixture of 10 times NR NOAEL doses
B
Likelihood for cleft phallus at PND16
VZ FIN PRO DEHP
C
Likelihood for hypospadias at PND47

Supplementary Table 1a. Statistical dose-effect descriptors for pooled single and mixture exposures – nipple retention and normalized AGD index

Substance	Dose Response Function						Effect doses (mg/kg/day)				Max. observed
	RM [a]	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	θ_{min}	$\hat{\theta}_{max}$	medium		low		mean effect
Nipple retention							ED6 [b]	[CI]	ED3 [b]	[CI]	
vinclozolin	G.Logit	-5.72	4.97	7.80	0	12	42.5	[36.0;45.9]	30.2	[25.9;33.2]	11.8
finasteride	Logit	3.25	2.09	-	0	12	0.028	[0.016;0.047]	0.008	[0.004;0.02]	9.5
DEHP	Logit	-4.22	1.32	-	0	12	1598	[612;11680]	234	[127;583]	5.2
prochloraz	BC-logit	-2.81	0.20	0.32	0	12	199	[99;714]	60.4	[34.0;93.1]	4.4
mixture	Logit	-4.38	3.09	-	0	13	23.3	[17.0;29.4]	10.6	[6.5;15.3]	11.6
AGD index (normalized to the controls)							ED60 [c]	[CI]	ED90 [c]	[CI]	
vinclozolin	Weibull	-9.97	4.94	-	0	1	76.1	[65.0;86.6]	36.5	[22.1;52.8]	25%
finasteride	Logit	-0.99	0.62	-	0	1	8.63	[1.83;94.7]	0.34	[0.19;0.57]	60%
DEHP	Weibull	-6.85	1.89	-	0	1	1945	[913; n.d.]	275	[41;1639]	86%
prochloraz	Logit	-12.00	4.95	-	0	1	219	[151;6314]	95.4	[39.2;147.7]	80%
mixture	Logit	-10.51	1.70	-	0	592	77.1	[59.4;99.8]	11.8	[4.3;34.7]	37%

[a] RM – Regression models as defined in (Scholze et al. 2001); $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3$ – Statistical estimates of model parameters, given for doses expressed as mg/kg/day (rounded values); $\hat{\theta}_{max}$ – upper model asymptote; θ_{min} – fixed lower model asymptote; [b] ED6, ED3 – Effect doses for 6 and 3 nipples, calculated from the respective dose response function; [c] ED60, ED90 – Effect doses for 60 % and 90 % normalized AGD index, calculated from the respective dose response function; CI – 95 % confidence intervals for mean effect doses given in mg/kg/day.

Supplementary Table 1b. Statistical dose-effect descriptors for pooled single and mixture exposures - organ weights and occurrences of malformation from male PND16 rat pups

Substance	Dose Response Function						Effect doses (mg/kg/day)				Max observed
	RM [a]	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	θ_{min}	$\hat{\theta}_{max}$	ED60 [b]	[CI]	ED90 [b]	[CI]	mean effect
Ventral Prostate (mg, normalized to the controls)											
vinclozolin	logit	-7.96	3.94	-	0	0.99	81.1	[66.9;97.4]	27.2	[14.4;47.7]	33%
finasteride	logit	-2.02	0.69	-	0	1.00	216.7	[69.3;1965.6]	0.54	[0.02;6.28]	67%
DEHP	G.logit I	-9.93	2.44	0.996	0	1.00	2169	[n.d;n.d.]	114.1	[12.7;426.2]	71%
prochloraz	logit	-8.58	3.31	-	0	0.99	292	[n.d.;n.d.]	79.2	[4.9;531.8]	80%
mixture	logit	-10.18	4.85	-	0	1.00	103.7	[78.9;130.5]	44.7	[13.9;79.3]	48%
LABC[c] (mg, normalized to the controls)											
vinclozolin	logit	-8.32	3.80	-	0	0.99	120.1	[95.4;190.8]	39.4	[16.0;73.6]	47%
finasteride	logit	-1.64	0.78	-	0	0.99	38.6	[10.8;651.8]	0.18	[0.01;7.86]	64%
DEHP	logit	-3.82	0.59	-	0	1.00	604000	[n.d.;n.d.]	619	[11;13337]	75%
prochloraz	logit	-9.55	4.13	-	0	1.00	164	[n.d;n.d.]	60.1	[15.8;113.7]	79%
mixture	logit	-9.76	4.53	-	0	1.02	118.9	[93.9;152.1]	51.0	[19.3;81.9]	56%
Malformation[d] (likelihood, %) at PND16							ED50 [e]	[CI]	ED10 [e]	[CI]	
vinclozolin	logit	-21.98	11.3	-	0	1	87.7	[78.5;103.2]	56.1	[45.8;74.4]	78%
finasteride	logit	-2.27	1.78	-	0	1	18.8	[7.2;62.5]	1.1	[0.3;7.5]	70%
mixture	Weibull	-14.68	7.39	-	0	1	37.5	[32.5;44.6]	25.5	[21.0;31.2]	92%

[a] RM – Regression models as defined in (Scholze et al. 2001); $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3$ – Statistical estimates of model parameters, given for doses expressed as mg/kg/day (rounded values); $\hat{\theta}_{max}$ – upper model asymptote; θ_{min} – fixed lower model asymptote; [b] ED60, ED90 – Effect doses for 60 % and 90 % normalized organ weights, calculated from the respective dose response function; CI – 95 % confidence intervals for mean effect doses given in mg/kg/day; n.d. – not determined, [c] Levator ani/bulbocavernosus muscles; [d] enlarged preputial clefts and hypospadias; [e] ED10, ED50 – Effect doses for a 10 % and 50 % likelihood of a malformation occurring, calculated from the respective dose response function.

Supplementary Table 1c. Occurrences of genital malformations at PND16 in male rat pups after administration of vinclozolin, finasteride, DEHP and prochloraz to pregnant rats from GD7 to PND16. Results from 2-3 independent studies are shown (number of pups and litters examined in brackets).

		Dose (mg/kg/day)								
vinclozolin	Controls	5	10	20	24.5	40	50	80	95.9	160
Study1	0% (40;13)	0% (24;5)	0% (21;6)	0% (32;7)	--	0% (19;7)	--	30.3% (33;8)	--	75% (12;6)
Study 2	0% (36;13)	--	--	--	0% (37;13)	--	--	--	77.8% (36;11)	--
Mixture study[a]	0% (31;13)	0% (18;5)	--	--	--	--	4.3% (23;7)	--	--	--

		Dose (mg/kg/day)					
finasteride	Controls	0.001	0.01	0.1	1	10	50
Study 1	0% (46;14)	0% (23;7)	0% (29;7)	5.6% (18;8)	8.7% (23;8)	35.3% (17;8)	69.6% (23;6)
Mixture study	0% (31;13)	--	0% (17;5)	0% (15;5)	--	--	--

		Dose (mg/kg/day)							
DEHP	Controls	3	10	15	30	100	300	600	900
Study1	0% (46;14)	--	0% (28;7)	--	0% (26;6)	0% (23;7)	4.8% (21;6)	0% (23;6)	0% (26;6)
Mixture study	0% (31;13)	0% (15;4)	--	0% (26;7)	0% (22;6)	--	--	--	--

		Dose (mg/kg/day)						
prochloraz	Controls	5	10	25	30	50	100	150
Study1	0% (40;13)	--	--	--	--	0% (29;8)	--	9.5% (21;6)
Study 2	0% (37;15)	0% (28;7)	0% (27;8)	0% (19;7)	--	0% (19;6)	0% (24;5)	--
Mixture study	0% (31;13)	0% (22;7)	--	0% (14;5)	--	0% (15;6)	--	--

a Doses run in parallel with the mixture experiment, study No 4 (see Table 1)

-- Doses not examined

Supplementary Table 2. Summary of the outcome of the mixture experiment, and the responses to the single chemicals tested in parallel with the mixture.

Group (dose in mg/kg/day)	# of litters	AGD index	Nipple retention	Right testis (mg)	Left Testis (mg)	Epididy-mides (mg)	Ventral Prostate (mg)	Seminal vesicle (mg)	LABC[e] (mg)	Bulbour Glands[f] (mg)	Adrenals (mg)	Kidneys (mg)	Liver (mg)	Thyroid Gland (mg)	malformation (%) PND16[g]	malformation (%) PND47[h]
Control	13	11.3±0.2	1.6 [0.9;2.5]	65.9±1.9	66.7±1.6	24.9±0.4	15.1±1.2	18.1±0.8	25.3±1.2	1.6±0.1	10.8±0.6	372±19	949±49	5.0±0.3	0[c]	0[c]
Mix1 (13.01)	16	**10.5±0.1[b]**	**3.7 [2.6;5.1][a]**	63.9±1.6	64.7±1.6	23.2±0.7	14.3±0.5	17.2±1.3	24.9±0.9	1.7±0.2	10.5±0.3	348±11	878±26	4.5±0.2	0[c]	0[c]
Mix2 (65.05)	10	**9.7±0.3[b]**	**10.1 [9.3;10.7][b]**	65.9±2.2	65.0±2.1	**22.0±0.9[a]**	**12.2±0.8[a]**	17.8±1.0	**18.5±2.6[a]**	**1.0±0.1[a]**	10.6±0.3	362±11	942±21	4.7±0.4	25.0 [13.6;41.5]	21.4 [7.1;49.4]
Mix3 (130.1)	12	**8.0±0.2[b]**	**11.6 [11.1;12.0][b]**	60.0±2.3	57.2±2.5	**21.2±1.0[b]**	**6.7±0.7[b]**	**12.2±1.8[a]**	**13.1±0.8[b]**	**0.5±0.2[b]**	10.3±0.4	335±20	870±32	4.4±0.3	92.1 [78.2;97.4]	100[c]
VZ (5)	6	11.1±0.5	2.7 [1.6;4.3]	58.6±1.4	59.5±1.1	21.9±2.1	12.6±0.9	15.7±1.3	21.4±2.4	1.1±0.3	10.5±0.4	356±26	826±49	4.4±0.1	0[c]	0[c]
VZ (50)	8	**10.3±0.3[b]**	**8.4 [6.9;9.6][b]**	63.7±4.4	63.8±3.9	**21.9±1.3[a]**	12.9±1.0	16.2±1.6	25.5±3.1	**0.5±0.1[a]**	12.0±1.1	373±43	993±95	4.6±0.5	4.3 [0.6;25.2]	12.5 [1.7;53.7]
FIN (0.01)	5	10.8±0.2	**4.4 [2.8;6.2][a]**	65.0±2.5	65.7±2.7	24.6±0.6	14.4±0.8	17.0±1.4	24.4±0.5	1.1±0.2	10.2±0.4	362±16	889±35	4.7±0.3	0[c]	0[c]
FIN (0.1)	6	**10.3±0.2[b]**	**8.7 [7.5;9.7][b]**	61.3±2.4	60.4±2.6	**22.1±0.9[a]**	15.0±2.0	16.4±1.4	25.6±1.8	0.8±0.3	10.0±0.6	364±23	869±39	4.7±0.5	0[c]	0[c]
DEHP (3)	5	11.1±0.6	1.8 [0.6;4.7]	61.8±1.1	61.9±1.7	22.8±1.5	13.7±0.9	17.0±1.7	24.0±1.1	1.4±0.3	10.1±0.5	326±7	852±34	5.3±0.3	0[c]	0[c]
DEHP (15)	7	11.2±0.2	1.7 [0.9;3.0]	63.2±2.2	63.3±2.0	24.0±1.0	15.1±0.8	14.3±1.1	23.8±1.1	1.6±0.1	10.7±0.7	358±22	910±36	5.0±0.2	0[c]	0[c]
DEHP (30)	7	11.5±0.3	3.1 [2.0;4.6]	68.3±1.9	68.5±1.9	27.0±0.7	17.0±1.0	17.3±1.9	24.5±1.3	1.8±0.2	10.9±0.7	378±13	1005±27	5.0±0.4	0[c]	0[c]
PZ (5)	7	11.5±0.2	2.9 [1.8;4.5]	65.4±2.9	65.3±2.2	23.6±0.9	15.1±1.4	20.3±1.3	25.2±0.7	1.4±0.2	10.5±0.6	352±17	922±40	4.5±0.2	0[c]	0[c]
PZ (25)	5	11.5±0.2	3.2 [2.3;4.4]	62.9±0.6	64.7±0.5	23.4±0.6	13.6±0.8	18.0±2.6	25.9±1.2	1.1±0.2	11.3±0.3	346±11	897±29	4.5±0.3	0[c]	0[c]
PZ (30)	7	11.3±0.4	**3.6 [2.2;5.4][a]**	73.3±4.5	73.5±3.6	26.3±2.2	15.4±1.2	15.1±1.1	23.7±1.9	1.3±0.2	11.9±0.7	377±13	989±53	5.8±0.8	0[c]	0[c]

Mix1, Mix2, Mix3: combinations of vinclozolin, finasteride, prochloraz and DEHP at the ratio of 5 : 0.01 : 5 : 3. VZ: vinclozolin, FIN: finasteride, PZ: prochloraz. Data represent means ± SEM, in the case of nipple retention and hypospadia data are means ± 95%CI

[a, b]: Statistically significantly different relative to controls ($p < 0.05$) and ($p < 0.01$), respectively. All statistically significant numbers are shown in bold.

[c]: Statistical analysis was not possible as all individual responses had the same value.

[e]: Levator ani/bulbocavernosus muscles; [f]: Bulbourethral glands; [g]: enlarged preputial clefts and hypospadias; [h]: severe hypospadias including exposure of the os penis and blind vaginal opening.

Supplementary Table 3. Comparison of NOAELs estimated for nipple retention in the present study with those currently used for regulatory purposes in human risk assessment

Compound	Our NOAEL (NR) mg/kg/day	Regulatory NOAEL mg/kg/day	Comments	References
DEHP	3	5	Based on effects on testes	EU RAR 2004
		Between 1 and 10	Based on effects on Leydig cells	NTP –CEHR 2005
Vinclozolin	5	4	Study of reproductive toxicity	RAR from http://www.inchem.org
		4.9	2. Generation study in rats	BASF Study
Prochloraz	5	3.7	Multigenerational reproductive toxicity in rats	JMPR 2001
Finasteride	0.01	0.10	Based on hypospadias	MERCK Propecia® (Finasteride) Prescribing information
		0.03	Based on nipple retention	
		0.003	Based on AGD	

DEHP:
EU RAR. EU-risk assessment of bis(2-ethylhexyl) phthalate (DEHP). Consolidated final report March 2004.

NTP-CERHR. Expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate 2005.

Vinclozolin:
BASF Studies Reproductive Toxicity Testing Two-Generation Reproduction
RAR from http://www.inchem.org

Prochloraz:
JMPR (Joint Meeting on Pesticide Residues) 2001

Finasteride:
Link to prescribing information from the producer MERCK:
http://www.merck.com/product/usa/pi_circulars/p/proscar.html